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Apolipoprotein A1 and polymorphisms

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Apolipoprotein A1 and Polymorphisms

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Abstract

The aim of the project was to detect the polymorphisms in the apo A1 gene, which might possibly be associated with the risk of atherosclerosis in claudicants. This aim was achieved by amplification of the three exons and four introns of the apo A1 gene and followed by sequencing of the amplicons. The annealing temperature estimated by gradient PCRS for the coding and non-coding regions was 60.5°C, 61.2°C, 62.5°C, 59.7°C, 58.1°C and 57.8°C for 5'flanking, exon2/intronB/exon3, exon 3, intron C, exon 4 and 3'flanking, respectively. Altogether, 138 PCRs were undertaken. It was not possible to detect any high confidence polymorphism in the present sample size which was further reduced due to failed sequencing reactions in some samples. 0nly 16 out of 60 samples were successful for sequencing reaction. None of them could be successfully sequenced with their both forward and reverse primers. Any true estimates of the allele and genotype frequencies of the mutations cannot be made from the observed small sample size. Also, depending on the sample size and the sequencing results, any inference as to how these mutations could be associated with artherosclerosis in claudicants could not be made.

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Introduction

1.1 Introduction

Oxidative stress can be described as the presence of excess of free radicals and the ability of body to neutralize them. ROS/RNS (reactive oxygen species/reactive nitrogen species) are present in living cells and tissues in very low concentration under normal physiological conditions (figure 1.1). The balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes decides their concentrations. Their balance is changed by redox regulation, either by an increase in RNS/ROS production or decrease in the activity of the antioxidant system (Turko, I. & Murad, F, 2002).

ROS/RNS can either be free radicals, reactive anions containing oxygen or molecules containing oxygen which have the ability to produce free radicals eg, hydroxyl radical($OH^{\cdot}$), peroxynitrite ($ONOO^{-}$) and hydrogen peroxide (H_2O_2). Superoxide ($O_2^{-\cdot}$) and other ROS are produced by oxidases and peroxidases in the body to perform useful functions. However, in disease states production increases over and above the requirements for normal metabolism which leads to the generation of potentially cytotoxic species. Superoxide is not itself very reactive but acts as a precursor for a number of ROS. These species modify the activity of antioxidant enzymes either by inactivation or leakage from cells, or by the release of metal ions from metalloproteins. The reactivity of ROS is increased when reduced with transition metal ions such as (Fe+2 or Cu+2) giving rise to hydroxyl radicals, or their interaction with a few haem proteins, or their reaction with myeloperoxidase which in turn forms hypochlorite, an oxidant which can damage proteins by producing chloramines which are long lasting (Rice-Evans, C & Bruckdorfer, K.R, 1995).

The major source of ROS amplification is the reaction of superoxide with nitric oxide (NO) producing a very potent oxidant, peroxynitrite. Its role has been discussed in detail in protein nitration below:

Figure 1.1 Oxidative Stress (Fiers, W. et al, 1999.).

Intracellular superoxide is produced by means of mitochondrial electron chain and cytosolic oxidases, such as NADPH/NADH oxidase or xanthine oxidase (Turko, I. & Murad, F, 2002). Aerobic respiration is thought to be the main source of ROS in the body.

Other sources are peroxisomal beta oxidation of fatty acids, microsomal cytochrome P450 metabolism of xenobiotic compounds, stimulation of phagocytosis by pathogens, arginine metabolism (Radi, R. 2004). ROS are present in very low concentration as compared to that of oxidizable substrate. Antioxidants which are found in plasma and soluble in lipoprotein include ascorbate, albumin, bilirubin, urate, tocopherols, heamopexin and carotenoids.

Nitric oxide (NO) plays a most important role in many physiological processes such as neurotransmission, inhibition of platelet adherence and aggression, blood pressure control, neutrophil aggregation, and blood clotting (Lupidi, G. et al 1999). In these conditions the concentration of NO keeps changing frequently in all parts of the body. NO is produced from L-arginine which acts as a substrate of three isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS or NOS-I) originally identified in brain and endothelial NOS (eNOS or NOS-II) present in endothelial cells. Both nNOS and eNOS require calcium and calmodulin as cofactors and generate low amounts of ˙NO (Turko, I. & Murad, F, 2002). A few pathological processes like reperfusion of ischaemic tissues stimulate NO production after induction of its third isoform, inducible NOS (iNOS or NOS-III), which is basically found in macrophages and does not require calcium or calmodulin as co factors (Colasanti, M. & Suzuki, H. 2000). This enzyme generates larger amounts of NO than the other two isoforms. This is thought to be the mechanism behind cytokine mediated cardiac contractile dysfunstion and development of cardiovascular disease.

One of the mechanisms by which excess NO can injure tissues is by its reaction with the ROS, superoxide radical (O_2^-) to give the main end product, the peroxynitrite anion ($ONOO^-$), which is a potent nitrating agent (Halliwell, B. 1997). However, the role of peroxynitrite to initiate tyrosine nitration in vivo has been controversial (Radi, R. 2004). The biological half life of peroxynitrite short (Lupidi, G. et al 1999). Being highly reactive it interacts with the proteins near the site of generation and cause the covalent modification of several amino acid residues in proteins, such as cysteine, methionine, tryptophan and tyrosine residues (Lupidi, G. et al 1999). This covalent modification generates 3-nitrotyrosine (3NT) which is used as a marker of oxidative damage (Aulak, K. 2003).

1.2 Protein Nitration

Nitration of the protein tyrosine is thought to be the footprint for in vivo production of free radical species (Turko, I. & Murad, F. 2002). It is a post-transational modification which occurs in a number of diseases. It alters protein structure, function and metabolism and produce dysfunctional changes. It may loose or gain an extra function but the general effect is inhibition. Nitration may prevent or activate the phosphorylation of tyrosine residues. Protein nitration is involved in disease initiation and progression (Turko, I. & Murad, F. 2002)**.** Most commonly, oxidative stress can lead to in vivo modification of proteins through nitration of tyrosine residues by RNS such as peroxynitrite (Galinanes, M. & Matata, B. 2002) and 3NT is the stable end product of such reactions (Shigenaga, M. et al, 1997).

Alternative pathways of protein nitration include oxidation of nitrite by peroxidases and hydrogen peroxide. Moreover, some transition metal dependent

mechanisms have come to light. They all result in oxidative damage through the nitration of tyrosine residues (Radi, R. 2004). Nitrite is known to be the substrate for haemoproteins when it is converted to nitrogen dioxide by myeloperoxidase, eosinophil peroxidase as well as other haemeproteins (cytochrome P450s, myoglobin), which act in tissue oxidant formation and accounts for nitration of tyrosine residues (Ischiropoulos, H. 2003). Also, the formation of nitrous acid (HNO_2) by nitrite acidification leads to the nitration of tyrosine residues in proteins. This reaction needs a lower pH (pH< 6.0) and the acid formed is able to nitrate protein slowly without requiring superoxide and hydrogen peroxide (Ischiropoulos, H. 2003).

Chemically, nitration is the addition of the bulky nitro group ($-NO_2$) onto one of the ortho carbons of the aromatic rings of tyrosine residues (Ischiropoulos, H. 2003). This lowers the pK_a of the phenolic group from about 10 to 7 and therefore disrupts the ability of tyrosine to function in electron-transfer reactions and maintenance of protein conformation (Tarpey, M. & Fridovich, I. 2001). Not all protein tyrosine residues undergo nitration and similarly not all proteins are nitrated. This has led to the view that nitration may be a normal physiological control mechanism as well as a pathophysiological event.

Tyrosine nitration has been observed as an irreversible process, however some studies have stated that protein nitration is reversible and may have a regulatory function, therefore enabling protein nitration to have a more dynamic physiological role. “Denitrase” activity has been suggested that may remove the nitro group from nitrotyrosine in proteins therefore displaying the possibility of a repair mechanism, thus signal transduction (Gow, A. *et al,* 1996).

Protein nitration has been postulated to function in cellular signalling events analogous to protein phosphorylation and dephosphorylation, resulting in kinase activation, which presumes the reversibility of tyrosine nitration (Tarpey, M. & Fridovich, I, 2001). It has been shown that peptides and proteins that are nitrated on tyrosine residues are poor substrates for tyrosine kinases, possibly due to interference between the nitration of tyrosine on the 3-position with the phosphorylation of the hydroxyl group on the 4-position (Kamisaki, Y. *et al,* 1998). Therefore the removal of the nitro group by "denitrase" could restore protein function and allow them to become substrates for tyrosine kinases and have important effects on cell signalling pathways (Kamisaki, Y. *et al,* 1998).

In pathophysiology, evidence suggests that most of the cardiovascular disease is the result of the high production of these free radicals and reactive species in the body and features the accumulation of nitrated proteins on one or other site depending on the type of disease such as cardiovascular inflammation, atherosclerosis, autoimmune myocarditis, heart failure, ischaemia reperfusion injury as well as many more (Turko, I. Murad, F. 2002). The pro and antiatherogenic role of NO has been broadly studied. Its pro atherogenic role can be described by the modification of lipids caused by RNS/ROS derived from NO metabolism. Protein tyrosine nitration was found to be associated with iNOS expression and confirmed iNOS positive in macrophage lesions at various stages of atherosclerosis. A study has revealed the tyrosine nitration of prostacyclin synthase on bovine atherosclerotic arteries on the very early early stage when arteries are thickened without any signs of necrosis and rupture of plaques.

Inactivation of prostacyclin synthase may predispose further platelet aggregation and thrombus formation. Intermittent claudication may be one of the consequences of severe atherosclerosis and protein nitration.

1.3 Intermittent Claudication (IC)

Claudication is basically a circulation problem which causes pain in the lower limbs while walking, as the muscles need more oxygen. The circulation becomes normal on resting. This condition features the presence of cholesterol and other fatty deposits in the arteries which narrows them and reduces the blood flow due to the presence of these atherosclerotic plaques (Tarpey, M. & Fridovich, I, 2001). The pain is experienced in the thigh and calf. It is known to be the symptom of peripheral arterial disease (PAD) and also known as “hardening of arteries” or atherosclerosis, which is a precursor to IC. Claudication pain is intermittent as it comes and goes. It prevails on exercise with the increased oxygen demand and tends to decrease when the person starts resting. In case of severe artery blockage the pain is experienced even in the rest condition. Partially clogged arteries are able to deliver enough blood and oxygen at rest but during exercise the need for oxygen outweighs the supply and causes pain (Stewart, A. & Lamont, P, 2001). ROS are potentially concerned in atherosclerosis by contributing to the oxidative damage of low density lipoprotein (LDL), which is a critical event, in the development of the atherosclerotic plaque (White, R *et al*, 2004). This oxidation takes place at almost all the sites of inflammation. One event is the formation of peroxynitrite which initiates oxidation and forms 3NT.

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1.4 Apolipoprotein A1

Protein constituents of lipoproteins are known as apolipoproteins. In plasma, they are found in high density lipoprotein (HDL) which functions to remove the cholesterol from the tissues and drain it back into the liver. It has been demonstrated that measurement of various forms of apolipoproteins can improve the prediction of risk of cardiovascular disease (Walldius, G & Junger, I, 2004). Lipoproteins are made up of a lipid core of cholesterol ester which is surrounded by a coat of cholesterol, phospholipids and Apolipoprotein (figure 1.2). Apo A1 is synthesised in the liver and small intestine and is the cofactor for lecithin cholesterol acyltransferase (LCAT) which is responsible for the syntheis of most of the plasma cholesteryl esters. The exact mechanism of action of apo A1 is not yet understood but it actively stimulates the cholesterol removal from cells and has an antioxidant ability which prevents some of the inflammatory damage in atherosclerosis. The anticlotting effect of apo A1 has been demonstrated by its isolation as a prostacyclin (PGI2) stabilising factor (Yui, Y *et al*, 1988).

Figure 1.2 Structure of apolipoprotein A1 (Gurgel, E.F. et al, *n.d*)

Apolipoprotein A occurs in two major forms, apo A1 and apo A2. But it is apo A1 which is found in abundance and plays a very important role in HDL metabolism. Apolipoprotein A1 is the chief constituent of HDL and acts as the primary acceptor of the unesterified cholesterol from the peripheral tissues (Eckardstein, A *et al*, 2001). It occurs as a single polypeptide chain of 243 amino acids residues (Sasaki, J. *et al,* 2000).

It has been reported that apo A1 not only inhibits the inititation and progression of atherosclerosis but also makes the pre-exisisting atherosclerotic

lesions regress (Sheng, Wu *et al*, 2005). Apo A1 and apo B carry cholesterol in the blood stream. But apo B is cholesterol donor found in low density proteins (LDL) and apo A1 is cholesterol scavenger found in HDL.

LDL cholesterol is a very critical feature in the development of atheroma. HDL is involved in its reverse transport to the liver and hence is Anti-atherogenic (Lupidi, G. *et al*, 1999). Cholesterol is an essential component of the cell. The bile acids are manufactured in the hepatocytes from cholesterol, and finally it is excreted from the body in the form of bile. Its presence must be controlled to maintain the body homeostasis. If the cells are unable to give up the free cholesterol (FC), it is stored as esterified cholesterol (EC). The cholesterol is transported to the cells as low density lipoproteins (LDL) (Aulak, K. *et al*, 2001). The LDL receptor (LDL-R) present on the cells is controlled by a negative feed back mechanism. Increased cholesterol level tends to suppress the transcription of LDL-R which in turn delays the LDL clearance. If the cholesterol level is decreased, the transcription is induced which maintains the feed back mechanism (Aulak, K. *et al*, 2001).

At the commencement of metabolism, a cholesterol-poor HDL particle, which has apo A1, acquires cholesterol and phospholipids from peripheral cells. This is facilitated by adenosine triphosphate-binding cassette protein-1 (ABCA1), beginning a process known as reverse cholesterol transport (RCT) (figure 1.3). Now, the cholesterol within the HDL particle is esterified by LCAT and this esterified cholesterol is ultimately taken up by the liver through the scavenger receptor typeB1 (Ballantyne and Nambi, 2004).

Cholesteryl ester transfer protein (CETP) replaces the cholesteryl ester in HDL by triglyceride from other proteins. Levels of apo A1 are strongly linked with those of HDL-cholesterol, and, as a result the expression of apo A1 may be responsible for determining the plasma level of HDL.

Figure 1.3 Schematic representation of the role of the ABCA1 lipid transporter in HDL biogenesis and reverse cholesterol transport. (Tontonoz and Mangelsdrof, 2005)

In addition to reverse cholesterol transport, HDL might protect against atherosclerosis by preventing oxidation of LDL and promoting the availability of nitric oxide, and modulating expression of endothelial cell adhesion molecules

(Ballantyne and Nambi, 2004). Yamato, K *et al*, 2005, suggested the possible links between HDL and apo A1 metabolism and atherosclerosis in patients with type 2-diabetes. Elevated serum LDL and depressed HDL in conjugation with elevated triglycerides (Tg), are well known risk factors for CVD. HDL-2 is the largest particle amongst all of HDL particles, which contains the most lipids in the core. And, pre β HDL is of the smallest size and active in taking up the peripheral cholesterol.

1.5 Mutants of Apolipoprotein A1

The gene that encodes apolipoprotein A1 is basically located on chromosome 11q23-q24 with two other apolipoprotein genes; apo C3 and apo A4 (figure 1.4). The coding region is in red and the untranslated region in blue.

Figure 1.4 Genomic region of apo A1 gene.

The apo A1 gene has three introns: IVS-1, IVS2 and IVS-3. The first interruption, IVS-1 is 197bp long and exists in the 5' untranslated region between bases 20 and 21 upstream of the codon for methionine (Met) which initiates translation. IVS-2 is 186bp long and occurs in the codon specifying amino acid 10 which is in the apo A1-prepeptide. The third sequence, IVS-3 is 588bp long and interrupts the codon specifying amino acid 43 of the mature protein. Most of the propeptide is present in exon 2 while exon 3 contains propeptide and amino terminus.

Exon 4 has codons for 200 amino acids which consist of COOH- terminal part of the molecule. It consists of six highly homologous 66bp long tandem DNA repeats. Upstream of the apo A1 transcription start site is a 7bp long AT-rich region which is probably the TATA box (Breslow, J.L, 1985).

Decreased concentrations of HDL cholesterol and apo A1 have been demonstrated because of the various mutations in apo A1. The mutations result into premature atherosclerosis, corneal opacities and xanthomatosis. The coronary risk was higher in cases where no plasma apo A1 was identified. But in a couple of cases no increased risk for myocardial infraction could be demonstrated (Assman sited in Rosseneu, 1995). In 1995, Lackner demonstrated a proband aws associated with developing early coronary artery disease with a deletion of apo A1, C3 and apo A4 gene.

Additionally, a Turkish girl was was found to have no detectable apo A1 when she was affected by HDL deficiency with xanthomas. Karathanasis described coronary artery disease associated with an inversion of 5.5 kb in the apo A1/C3 gene. The truncation of apo A1 at residue 230 leads to corneal opacity and LCAT deficiency. The deletion of residues 145-160 results in HDL deficiency and xanthomas. A deletion in intron 2 causes coronary heart disease. Defects in this gene are linked with HDL deficiencies, including tangier disease, and with systemic non neuropathic amyloidosis.

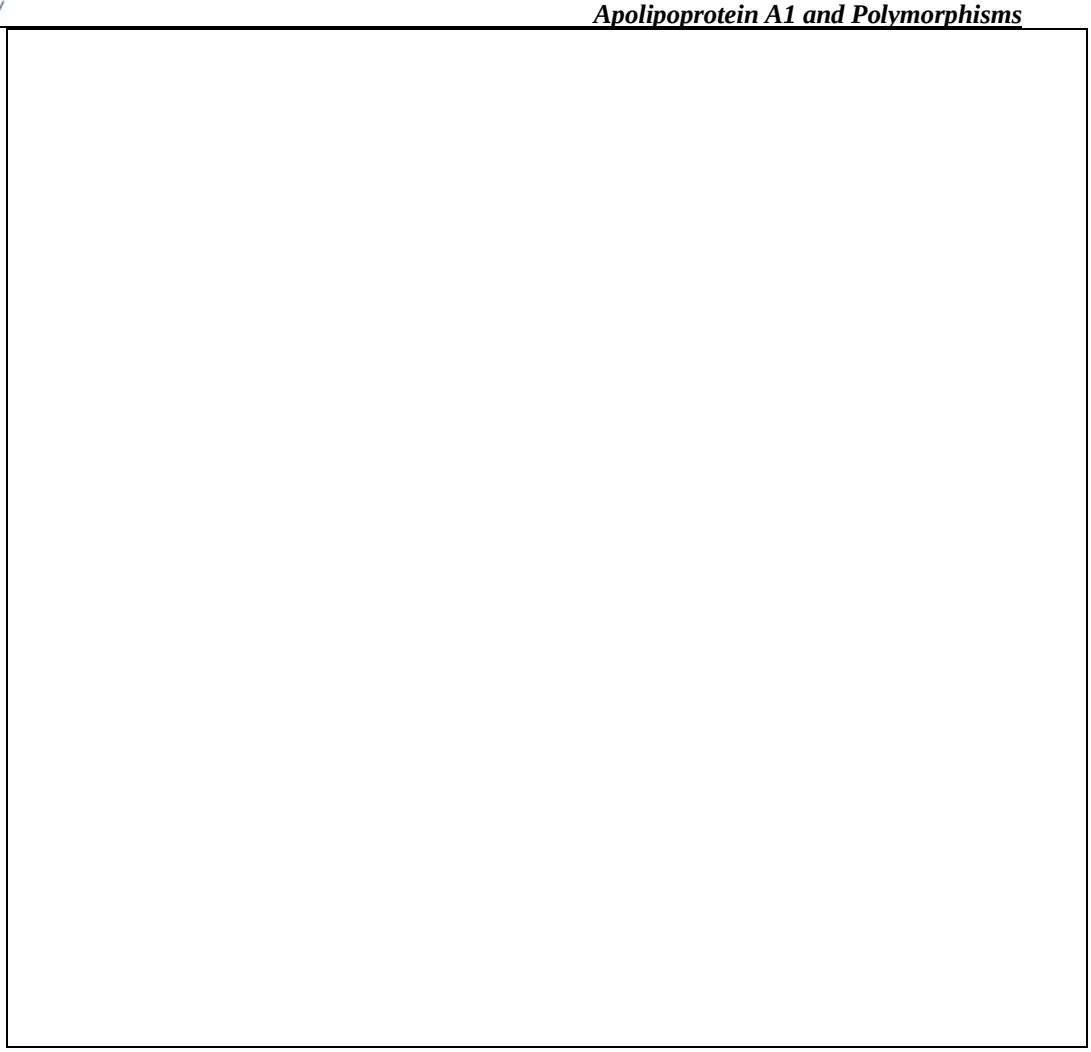

Figure 1.5 Mutations in apo A1 and functional implications (Rosseneu 1995).

Apo a1 Milano is an apo a1 mutant which was found in an Italian community from *Limone* on the shore of Lake Garda. The prominent feature of this molecular abnormality was hypoalphalipoproteinemia but without atherosclerosis or any risk of coronary heart disease. The presence of this mutation allowed the existence of an additional cysteine bridge which makes possible homodimer or heterodimer formation with apo a2. This mutation leads in some cases to impaired cholesterol esterification in HDL together with decreased LCAT activity.

Despite very low HDL cholesterol levels, no premature vascular disease has been found in apo A1 (Milano) carriers (Parolini C. *et.al, 2005*). A single amino acid mutation was found during an electrophoretic mutation screening of 32,000 new borns from Germany. They were presnt in the primary structure of apoA1 and are P165-R, P143-R, and K107 (Eckardstein, A. *et al*, 1990). A naturally occurring point mutation, V156E, is linked with extremely low plasma apo A1 and HDL levels and coronary heart disease (Kyun, H. and Jonas, A., 2000). HDL might protect against atherosclerosis by preventing low density lipoprotein oxidation and promoting the availability of NO (Ballantyne and Nambi, 2004).

The extent of atherosclerosis is determined by the size of lipoprotein particles, small LDL particles being more atherogenic and the patients have quite a few of the large HDL particles as compared to the healthy individual, and the severity of the disease increases as their number increases. (Gualberto R. et al) The reason behind the size variation and its effects has not yet been understood. It has been reported that a genetic variant in the apo A1 promoter alters the response of small and large HDL particle distribution to exercise training (Kouji, K. et al). A 75G/A single nucleotide polymorphism (SNP) has been demonstrated in apo A1 gene which is linked with HDL-C concentrations to dietary changes in polyunsaturated fat intake. 53 subjects were found to be G homozygotes (G/G) and 22 were A carriers (A/G and AA), when these 75 subjects were genotyped for 75G/A SNP. This genetic variation at the apo A1 gene promoter is linked with HDL redistribution resulting from exercise training (Ruano, G. *et. al,* 2006). In a recent study apo A1 has been demonstrated as a selective target for myeloperoxidase-catalyzed oxidation modification in human atheroma (Zheng L. *et al*, 2004).

HDL islolated from human atherosclerotic lesions and the blood of the patients with coronary artery disease were found to have elevated levels of 3-chlorotyrosine. Moreover, Tyr-192 was reported to be the major site which is chlorinated in apo A1 which is directed by lysine residues. As a result, the chlorinated apo A1 loses its facility to promote cholesterol efflux from cells by the ABCA1 pathway. On the other hand, methionine residues inhibit this chlorination and act as a local protein bound antioxidants. So, the combination of tyrosine chlorination and methionine oxidation might help apo A1 to maintain its functional state. They also suggested the modification of a limited number of amino acids occur during the oxidative damage of apo A1 and the oxidation-resistant forms of apo A1 have enhanced anti atherogenic activity in vivo (Shao, B. *et al*, 2006).

Mutations in ABCA1, which functions as a cholesterol efflux regulatory protein **(**Nan, W. *et al*, 2001**),** causes very low concentrations of HDL-C and apo a1 resulting in Tangier disease. The LCAT and the apo a1 were studied in unrelated French Canadian probands by direct sequencing to analyse the molecular causes of severe HDL cholesterol deficiency. A novel nonsense apo a1 mutation known as E136 was detected in a few subjects. They observed the segregation of the apo a1 locus and the defined mutation is a cause of HDL-C deficiency and related with premature coronary artery disease in the specified population. (Zari, D*. et al,* 2005)

Hoving, Gk. et al has demonstrated a novel mutation, L178P, in the apo A1 gene which leads to an endothelial dysfunction, increased arterial wall thickness

and finally premature coronary artey disease. Due to a limited number of carriers of the apo A1 defects, data on the consequences of such defects have remaines injustified and inconclusive.

1.6 Current project

Mercer, S. at Coventry University, in her previous study of claudicant patient's plasma, found some of the proteins nitrated including apo A1, which actually helps in removal of cholesterol and other fatty deposits from the tissues. The subjects were patients at a clinic at New Cross Hospital, Wolverhampton and the controls were university students and staff members. The nitration of ApoA1 in claudicant patients could possibly be because of mutations in ApoA1 which increases their risk of predisposition to atherosclerosis. It was therefore necessary to know the spectrum of mutations in ApoA1 gene in normal healthy control population to draw any inferences about association of mutations in ApoA1 with artherosclerosis in claudicants. With this broader aim, the present study focussed on investigating mutation profile of ApoA1 gene in normal healthy controls. The possibility was raised that claudicants may have a mutation in the apo A1 gene which predisposes them to atherosclerosis. Sequence information would be necessary to determine this and the current project was to establish the protocols in the DNA of control subjects. This is achieved by the amplification of the three main exons and three introns along with the 5' and 3' flanking regions, followed by sequencing of the amplicons. PCR and fluorescent DNA sequencing were the only methods used in the project.

Chapter 2
Methodology

2.1 Sample Collection

To ensure the efficiency of the PCR process, the saliva samples were collected from only one subject. Afterwards eight samples from different subjects were gathered by spitting in a tube. The sample volume was 2 ml and all of them were double-blind. Saliva sample was stored at 4°C as soon as they were collected. Samples which were to be used again were stored frozen at -80°C. All samples were processed as described below.

2.2 DNA Extraction from Sample

The Generation DNA purification system with the Generation Capture Column Kit from Gentra Systems was used for DNA-purification. The dry block heater was pre heated to 99°C. A Generation Capture Column contained in a blue Waste Collection Tube, an additional blue Waste Collection Tube and one clear DNA Collection Tube was assembled to process each sample. 200µl well mixed sample was inserted into the Capture Column whilst gently touching the centre of the matrix with the pipette. Samples were permitted to absorb at room temperature for at least one minute or up to an hour. The recommended time is 15 minutes. Afterwards, 400µl DNA Purification Solution (Solution 1) was added and incubated for one minute at room temperature. Solution started draining into the Waste Collection Tube during the incubation time. The sample was then centrifuged for 10 seconds at 12,000 x g. A waste volume of 600 µl was collected in the blue Waste Collection tube.

The Generation Capture Tube was transferred to the second blue Waste Collection tube. Another volume of 400 µl DNA Purification Solution (Solution 1) was added and incubated for one minute at room temperature. The centrifugation was repeated in the same manner as earlier. A waste of 400 µl was collected. The matrix was white or nearly white. 200 µl of DNA Elution Solution (Solution 2) was added and no incubation was done at this point. After centrifugation as described above 200 µl waste volume was collected. The matrix containing the purified DNA was white.

2.3 Sample Elution

The General Capture Column was transferred to a clear DNA Collection Tube and the second blue Waste Collection Tube containing 600 µl was discarded.

200 µl DNA Elution Solution (Solution 2) was added was allowed to incubate in a dry block heater pre heated to 99°C. The tubes were placed in such a manner that the Generation Capture Column was completely enclosed within the heat block to ensure proper heating of the sample. Immediately after heating, the sample was centrifuged for 20 seconds at 12,000 x g to free the purified DNA from the Generation Capture Column. The collected 200 µl DNA solution appeared clear. The Generation Capture Column was now discarded. The purified DNA was now ready for analysis.

2.4 DNA Storage

Purified DNA was stored for up to three months at 4°C. For long term storage, it was stored at –20°C.

2.5 DNA Quantification

Nucleic acid samples require purification prior to measurement to ensure accurate results. DNA isloated from organisms is tightly bound to the protein present within. It is not always possible to get rid of the protein component. To determine the concentration and purity of the DNA solution, the absorbance of UV light is measured in a spectrophotometer. Both DNA and protein absorb UV light and give different absorbance maxima, which are at 260 nm and 280 nm respectively. In an absorption spectrum, both curves slightly overlap in the areas including 260 nm and 280 nm. Absorbance at 260 nm is mainly due to the DNA but a little bit by the protein. At 280 nm it is the other way round. By dividing the two absorbance values the purity of the DNA solution is calculated. If the DNA solution is substantially free of protein, the 260/280 nm ratio will be high . A ratio of ~1.8 is generally accepted as

“Pure” for DNA; a ratio of ~2.0 is generally accepted as “pure” for RNA. Small changes in the pH of the solution will cause the 260/280 nm ratio to vary. Acidic solutions will under represent while the basic solutions will over represent this ratio by 0.2-0.3 (*Technical Support Bulletin, 2007*). The 260/230 nm ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for “pure” nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2(*Technical Support Bulletin, 2007*). If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.

Nanodrop, a small scale spectrophotometer was used for the DNA quantification. The apparatus is a cuvette-less fibre optic spectrophotometer with a 1mm light path which allows it to utilize even very small volumes of sample(1-2µl). The concentration and the purity of the sample were determined with this method. 1µl of DNA sample was positioned on the Nanodrop platform and an absorption spectrum from 320 nm to 230 nm was measured.

2.6 Polymerase Chain Reaction of ALU-TPA

The human genome contains a number of repetitive DNA sequences. These occur as tandem repeats (satellites, micro satellites or minisatellites) or as dispersed repeats (transposable elements), including LINEs (long interspersed nuclear elements) and SINEs (short interspersed nuclear elements). One class of SINE found in human genome is the ALU element.

In this experiment, the polymerase chain reaction (PCR) is used to amplify a nucleotide sequence from chromosome 8 to look for an insertion of a short DNA sequence called Alu within the tissue plasminogen activator (TPA) gene. These sequences are 280-bp long and are named Alu as they contain the recognition sequence for the restriction enzyme AluI. There are about 1 million Alu elements in the human genome, and they are known to have arisen very early in the course of human evolution. However some Alu elements have subsequently been lost from the genome and the modern human population frequently shows dimorphism (either Alu is present or absent) for particular Alu elements.

Alu elements analysed by PCR using primers complementary to unique DNA sequences flank (lie either side of) the Alu sequence. The length of the PCR product indicates whether Alu is present or absent. One such Alu element, called TPA-25, is found within an intron of the tissue plasminogen activator gene. This insertion is dimorphic. In this experiment, oligonucleotide primers, flanking the insertion site, are used to amplify a 400-bp fragment when TPA-25 is present and a 100-bp fragment when it is absent. Each of the three possible genotypes—homozygous for the presence of TPA-25 (400-bp fragment only), homozygous for the absence of TPA-25 (100-bp fragment only), and heterozygous (400-bp and 100-bp fragments) are distinguished following electrophoresis in agarose gels.

To test the efficacy of the DNA extraction and PCR process, PCR on an ALU element in an obtained human genome sample was carried out during the first week of experimentation. It confirms the performance of the sample and PCR buffer. The latter contains:

10mM KCl,

10mM $(NH_4)2SO_4$,

20mM Tris HCl,pH 8.8,

2mM $MgSO_4$,

0.01% TritonX-100,

Furthermore, a forward primer TPA25F 0.5µM (5’ GAT AGA GTT CCG TAA CAG GAC AGC T 3’), a reverse primer TPA25R 0.5µM (5’CCC CAC CCT AGG AGA ACT TCT CTT T 3’) and dNTPs 200µM each were added to the buffer to make the master mix. If they are used in excess, they may reduce the fidelity. Also, the freeze/thaw cycle is destructive. Hence, dNTPs, templates and *Taq*

polymerase (source: *Invitogen***)** were aliquoted on arrival. The tubes to be used in the experiment were allowed to incubate at 95°C for 5 minutes. Finally 2µl *Taq* polymerase (1 unit) was added to each tube and mixed.

Subsequently, the tubes were set in the thermal cycler for amplification. The conditions used for the process were 30 cycles at 94°C for 1 min, 58°C for 2 min, and 72°C for 2 min, and a concluding incubation at 72°C for 8 minutes. The PCR products obtained were analysed by gel electrophoresis. Free Mg^{2+} are required by *Taq* polymerase for its performance. The amount of free Mg^{2+} can be reduced by template DNA, chelating agents (e.g. EDTA), dNTPs (0.8mM $MgCl_2$ complexes with 0.2mM dNTP) and protein. So 1-4mM was tested for each primer set. $MgCl_2$ solution tends to make up a concentration gradient when frozen, so it was defrosted completely and vortexed before use.

2.7 Polymerase Chain Reaction (PCR) of the selected regions of the Apo A1 gene

Primers were found for all the exons and introns of the candidate gene. Web Primer, was used to define the primer pairs. The primer pair should not anneal with each other in any condition and have the reasonable G/C contents (<40%). Blast from NCBI was used to analyse the PCR fragment matches with the original gene sequence. Once the primer pairs were selected for all the exons and introns, they were ordered from Invitrogen. The PCR amplification of gene specific regions was carried out in 25 µl of reaction volume by using 25- 150 ng genomic DNA as a target. A master-mix (MM) was made to run every PCR. The composition of the PCR master mix(100µl) is listed below in Table 3.

Reagent	Stock Concentration	Final Concentration	Master Mix (100µl)
Sterile Water	-	-	84µl
PCR Buffer	10x	1/10 dilution	2.5µl
dNTP	10mM	200µM	2.3µl
$MgCl_2$	25mM	1mM	2µl
Primer A (reverse)	0.5µM	10.5pmol/µl	4.8µl
Primer B (forward)	0.5µM	12pmol	4.8µl

Table 1: Components of the reaction mixture

All the reaction mixtures were set up in an area separate from that used for DNA preparation or PCR product analysis. The solutions were completely mixed before use in order to avoid any localised concentration of salts.

The master mix typically contains all of the components for PCR excluding the template DNA. A volume of master mix was prepared which was 10% greater than that required for the total number PCR assays to be performed. A negative control (without template DNA) was always included in the PCR reaction. This was done to check if there is any contamination in the template DNA.

The master mix was mixed properly and dispensed in appropriate volumes into the PCR tubes. The template DNA was added to the individual tubes containing the master mix. Thermal cycling was performed on PTC-100 Programmable Thermal Controller (*Eppendrof* **),** using the conditions: Initial denaturation at 94°C for 1 min,

30 cycles of 94°C for 1 minute. Annealing at a temperature(different for the gene regions amplified) for 2 min and a final extension at 72°C for 5 minutes.

For the “hot start” PCR, the tubes were incubated for 5 minutes just after the centrifugation. One unit (2µl) of taq polymerase was added after the hot start and centrifuged. The PCR program was started and the PCR tubes were added once the thermal cycle reached the initial 94°C. After amplification, samples were stored overnight at -20°C for long term storage. The primer sequences used with the standardized PCR conditions are listed in the table below.

Apo A1 Gene regions	Primers Used	Amplicon Size (bp)
5’ Flanking	Fwd - TCTCCCTGGAATGCTGGTG Rev- TGTTGCTGCTCACTGGTCCT	330
Exon1- Intron A	Fwd- ACATTGCCAGGACCAGTGA Rev- TTCATCCTGAAGGGCCGT	348
Exon2-IntB-Exon3	Fwd- ATGCTGAAGGCACCCCACT Rev- CTGGGTCCTTACTTTAGCT	465
Exon 3	Fwd- ACAGCTGGCCTGATCTGGGT Rev- GGCTGGGTCCTTACTTTAGCT	209
Intron C	Fwd- GCTAAAGTAAGGACCCAGCCT Rev- AGGGTGAGACAGAAGGGTTGA	591

Exon 4	Fwd- AGCCCTCAACCCTTCTGTCT Rev- TTTATTCTGAGCACCGGGAA	674
3'Flanking	Fwd- TCAACACCCAGTGAGGCG Rev- CCTTGAGCCCCTTTCA	364

Table 2: Primers used for the standardised PCR reactions

2.8 Primer design

The primers were designed and ordered by the supervisory team. A computer programme called 'Web Primer' (Stanford University,California.) was used to design primers for the PCRs undertaken. The length and the content of the primers were taken into consideration. The primer pairs do not anneal with each other and have a reasonable G/C content, which is less than 40%. Web Primer is to be found on http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer. The c-DNA sequence was used without the polyA tail.

The polyA tail prevents the program finding a good primer pair because it will look for primer sequences with some minimum value of G/C content- not 0% as it would be the case for oligoT.

The cDNA sequence is submitted. The search was started within upto 50 bases from the 5' end and 3' ends. The best pair is examined and copied to the clipboard and edited in MS WORD. It can be analysed by BLAST, where exactly the PCR fragment matches on the original DNA. After searching for all the exons and introns, the primer pairs were ordered from *Invitrogen*.

The homepage http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi was used to find the m-RNA sequences of Apo A1 and as well as the gene sequence.

To locate any variation in the sequences of Apo A1 exons, restriction enzymes are needed. First of all, the cDNA sequence of Apo A1 is needed. For finding this sequence NCBI ENTRZ was used (www.ncbi.nlm.nih.gov/Entrez). The nucleotide sequence database could be browsed for the complete Apo A1 m-RNA entry by searching for "Homo-sapiens Apolipoprotein A1 m-RNA" or for the GI number 4557320. Once the correct m-RNA is found, the FASTA report display is required. This sequence is used for the restriction analysis. Afterwards, Web-Cutter (www.firstmarket.com/cutter/cut2.html) was used to find restriction enzymes. By following the instructions on the homepage all endonuclease were displayed.

2.9 Optimisation of PCR

The PCR conditions for ALU TPA were derived from the previous studies held in the university laboratory. Initially similar PCR conditions were used for the Apo A1 gene and gradient PCR's were designed later to find out the correct annealing temperature for the specific regions of the gene to be amplified.

In the beginning, none of the PCRs seemed to be working. The primer concentration was lowered to one tenth and one fifth . But no positive outcomes were gained. The control sample (without DNA template) was run with every PCR to ensure there was no contamination in the samples. Earlier, the control sample was found contaminated for some PCRs as it consistently showed a product band.

To overcome this, fresh reaction mixture was prepared and the reaction was done again. The DNA concentration was different for every sample. Samples with high concentration were diluted before use.

Gradient PCR with a annealing temperature gradient of 57±7°C was set up to find out the correct annealing temperature for the three exons of APO A1 gene. The temperature range in the PCR machine counting from first well till twelfth well was between 49.9°C and 64.5°C (TABLE). The PCR program is shown in TABLE.

Well	1	2	3	4	5	6	7	8	9	10	11	12
Temperature[°C]	49.9	50.1	50.8	52.2	53.8	57.8	58.1	59.7	60.5	61.2	62.5	63.2

Table 3: The Temperature in each well in a gradient PCR machine

Step	Temperature	Time	Cycles
1	94°C	1 min	30x
2	57°C Midpoint Gradient R= 2°C /s G= ±7ºC	2 min	

3	72°C	2 min	
4	72°C	5 min	1x
5	22°C	Hold	

Table 4: PCR program for APO A1; G= gradient 57°C ±7°C; R= ramp time

2.10 Gel Electrophoresis

After the PCR, gel electrophoresis was performed. Initially, for Alu PCR products, 2µl ethidium bromide (5mg/ml) was added to 35ml of 2% molten agarose. This molten agarose was then poured into gel apparatus and left to set on the bench. Once it is set, the running buffer (1 x TBE) was dispensed on top of the gel. TBE (Tris-Borate-EDTA) is a buffer solution which consists of Tris base, boric acid, EDTA and water.

Bromophenol blue loading buffer (10µl) was added to each PCR tube, mixed and centrifuged. The sample DNA was loaded into the wells of the gel. The power pack Shandon Vokam 400 was used and a current of 50mA was passed through the gel, until the bromophenol blue reached close to the anode. Furthermore, for the electrophoresis of the apo A1 amplicons a kit from Cleaver Scientific Ltd Multi Sub Horizontal Agarose Gel Electrophoresis Systems was used. The gel was then stained in EtBr (final concentration of 0.5 g/ml) for one hour and visualized under

UV illumination. A marker (table 2) was loaded on each side of the gel using 1µl of marker, 9 µl sterile water and 5 µl bromophenol blue for one well.

Ethidium bromide is known to be a potent mutagen. Then all the solutions and gel containing EtBr were handled very carefully. A lab coat and the gloves were worn worn all the time during the experiment and the process waste and contaminated solutions were disposed of in an activated charcoal bucket.

PCR marker		
Fragment	Base Pairs	DNA Mass
1	766	62ng
2	500	40ng
3	300	48ng
4	150	61ng
5	50	89ng

Table 5: PCR marker used for Gel-electrophoresis

2.11 Sequencing

The Plasmid to Profile service of The Functional Genomics Laboratory in the University of Birmingham provides a custom sequencing service based on fluorescent Sanger sequencing. The amplicons were isolated using a recommended kit, quantified and sent with the primers to the laboratory. The amount of PCR products used for sequencing was 5 – 10 ng. The products were

split into two tubes and 3.2 pmol of either forward or reverse primer was added. Finally, the samples were sent to the laboratory for sequencing. PCR amplified samples were treated with Exonuclease-I and Shrimp alkaline phosphatase enzyme (1X SAP buffer, 1U of SAP enzyme and 0.5U Exo-I enzyme). Mixture was incubated at 37°C for one and a half hour and enzyme was inactivated at 70°C for 15 min. The automated cycle sequencing of PCR amplified samples was carried out with specific primers used for PCR-amplification and universal sequencing primers using dye terminators (ABI PRISM Big Dye terminator cycle sequencing ready reaction kit). Ten microliter of Sequencing reaction was prepared (1X buffer, 0.125X Big Dye V 3.0 Ready Reaction mix, 0.4µM of primer and 10ng of well quantified sample) and pipetted into each well of a 96 well plate and cycle sequencing was performed under standard conditions as follows.

Rapid thermal ramp to 96°C

96°C for 10 seconds

Rapid thermal ramp to 50°C

50°C for 5 seconds

Rapid thermal ramp to 60°C

60°C for 4 min

Step 2 to 6 were repeated for 25 cycles

Samples were kept at 4°C until precipitation. 95% of ethanol was added to each well at a final concentration of 60 ± 3 % and sealed with strip caps. The plate was inverted few times to mix properly and left at RT for 15 min to precipitate the extension products. Plates were centrifuged at 2000x g for 20 min. Supernatants

was discarded by inverting the plate onto a paper towel. The pellets were washed with 150µl of 70% ethanol and centrifuged at 2000x g for 10 min. Washing with 70% alcohol was repeated once more.

Supernatant was removed by inverting the plates onto a paper towel. Plates were centrifuged in inverted position once at 700x g for 1 min to remove the residual ethanol. 10 µl of HiDi formamide was added to each well and samples were denatured at 95ºC for 4 min. and chilled immediately. Plates were placed on an AutoSampler for sequencing. The samples were electrophoresed on an ABI Prism 3100 Avant Genetic Analyser.

Fluroscence electropherogram results were obtained by e mail and were visualized with the CHROMAS program and compared with CLUSTAL W.

Chapter 3
PCR Results

3.1 PCR primer selection with Web primer

The primers were designed for all the three exons and four introns of the apo A1 gene using Web Primer program. The gene sequence of apo A1 is given below. Various colour patterns have been used to distinguish the primer sequences used to amplify different regions of apo A1 gene. The forward and reverse arrows indicate the forward and reverse primers used to amplify the respective regions.

5' END

1 tctccctgga atgctggtgg tgggggaggc agtctccttg gtggaggagt cccagcgtcc

61 ctcccctccc ctcctctgcc aacacaatgg acaatggcaa ctgcccacac actcccatgg

5' Flanking

121 aggggaagg gatgagtgca gggaaccccg accccacccg ggagacctgc aagcctgcag

181 acactcccct cccgccccca ctgaaccctt gacccctgcc ctgcagcccc cgcagcttgc

24 tgtttgccca ctctatttgc ccagccccag ggacagagct gatccttgaa ctcttaagtt

301 ccacattgcc aggaccagtg agcagcaaca gggccggggc tgggcttatc agcctcccag

Exon 1

361 cccagaccct ggctgcagac ataaataggc cctgcaagag ctggctgctt agagactgcg

421 agaaggaggt gcgtcctgct gcctgccccg gtcactctgg ctccccagct

Intron A

471 caaggttcag gccttgcccc aggccgggcc tctgggtacc tgaggtcttc tcccgctctg

531 tgcccttctc ctcacctggc tgcaatgagt gggggagcac ggggcttctg catgctgaag

Exon 2

591 gcaccccact cagccaggcc cttcttctcc tccaggtccc ccacggccct tcaggatgaa

651 agctgcggtg ctgaccttgg ccgtgctctt cctgacgggt aggtgtcccc taacctaggg

Intron B

711 agccaaccat cgggggggctt tctccctaaa tccccgtggc ccaccctcct gggcagaggc

771 agcaggtttc tcactggccc cctctccccc acctccaagc ttggcctttc ggctcagatc

831 tcagcccaca gctggcctga tctgggtctc ccctcccacc ctcagggagc caggctcggc

Exon 3

891 atttctggca gcaagatgaa ccccccccaga gcccctggga tcgagtgaag gacctggcca

951 ctgtgtacgt ggatgtgctc aaagacagcg gcagagacta tgtgtcccag tttgaaggct

1011 ccgccttggg aaaacagcta aagtaaggac ccagcctggg gttgagggca ggggtagggg

1071 gcagaggcct gtgggatgat gttgaagcca gactggccga gtcctcacct aatatctgat

1131 gagctgggcc ccacagatgg tctggatgga gaaactggaa tgggatctcc aggcagggtc

Intron C

1191 acagcccatg tcccctgcaa aggacagacc agggctgccc gatgcgtgat cacagagcca

1251 cattgtgcct gcaagtgtag caagcccctt tcccttcttc accacctcct ctgctcctgc

1311 ccagcaagac tgtgggctgt cttcggagag gagaatgcgc tggaggcata gaagcgaggt

1371 ccttcaaggg cccactttgg agaccaacgt aactgggcac tagtcccagc tctgtctcct

1431 ttttagctcc tctctgtgcc tcggtccagc tgcacaacgg ggcatggcct ggcggggcag

1491 gggtgttggt tgagagtgta ctggaaatgc taggccactg cacctccgcg gacaggtgtc

1551 acccagggct caccctgat aggctggggc gctgggaggc cagccctcaa cccttctgtc

1611 tcaccctcca gcctaaagct ccttgacaac tgggacagcg tgacctccac cttcagcaag

1671 ctgcgcgaac agctcggccc tgtgacccag gagttctggg ataacctgga aaaggagaca

1731 gagggcctga ggcaggagat gagcaaggat ctggaggagg tgaaggccaa ggtgcagccc

1791 tacctggacg acttccagaa gaagtggcag gaggagatgg agctctaccg ccagaaggtg

Exon 4

1851 gagccgctgc gcgcagagct ccaagagggc gcgcgccaga agctgcacga gctgcaagag

1911 aagctgagcc cactgggcga ggagatgcgc gaccgcgcgc gcgcccatgt ggacgcgctg

1971 cgcacgcatc tggcccccta cagcgacgag ctgcgccagc gcttggccgc gcgccttgag

2031 gctctcaagg agaacggcgg cgccagactg gccgagtacc acgccaaggc caccgagcat

2091 ctgagcacgc tcagcgagaa ggccaagccc gcgctcgagg acctccgcca aggcctgctg

2151 cccgtgctgg agagcttcaa ggtcagcttc ctgagcgctc tcgaggagta cactaagaag

2211 ctcaacaccc agtgaggcgc ccgccgccgc ccccccttccc ggtgctcaga ataaacgttt

2271 ccaaagtggg aagcagcttc tttcttttgg gagaatagag gggggtgcgg ggacatccgg

2331 gggagcccgg gtggggcctt tggccctgga gcagggactt cctgccggat ctcaacaact

3’ Flanking

2391 ccgtgcccag actggacgtc ttagggccaa gatcgacgtt ggaggacctg ctggacgcct

2451 ggctgcttac gagtgaggga gtagagtctg ccttagcaag gctcaagtag aaaggaagtc

2511 acagcggacc aggcaaagcc acagacaatc caaggccagg tgccctgaaa ggggctcaaa

2571 caaggcctgc

3' END

→ 5' flanking primers; 19merF, 20merR 330bp amplicon

→ Exon 1 / Intron A; 19merF, 18merR 348bp amplicon

→ Exon 2 / 3 Intron B; 18merF, 21merR 465bp amplicon

→ Exon 3; 20merF, 21merR 209bp amplicon

→ Intron C; 21merF, 21merR 591bp amplicon

→ Exon 4; 21merF, 20merR 674bp amplicon

→ 3' flanking; 18merF, 20merR 364bp amplicon

Figure 3.1 Gene sequence of human apolipoprotein A1. bp = base pairs. 18-21mer F/R = Forward or Reverse primer.

3.2 DNA purification

The DNA samples were isolated from university students and staff members. The samples were quantified using the Nanodrop spectrophotometer. The best results with the polymerase chain reaction can be achieved using 50 -150 ng of template. The ratio of absorbance reading at 260/230 nm and 260/280 nm was measured. The samples used in the procedure were in the concentration range 24.3 ng/µl to 150.7ng/µl. The samples with high concentration were diluted.

The 260/280 ratio othe samples signifies that the DNA is not pure, moreover, the ratio is also far from the value of 2 at 260/280 nm. The samples were not necessarily the same for every PCR.

Sample ID	Amount (ng/µl)	260/230 nm	260/280 nm
A1	104.0	0.99	1.89
A2	61.6	0.38	1.51
A3	98.5	0.65	1.67
A4	24.3	0.44	1.47
A5	37.0	0.29	1.31
A6	53.7	0.37	1.36
A7	72.9	0.69	1.72
A8	150.7	0.40	1.43
A9	85.4	0.35	1.47

Table 6: DNA extraction results

3.3 Alu TPA PCR Results

The results for Alu PCR are shown in the figure 3.1. This control PCR was done to ensure the quality of DNA templates and the reaction mix. Five samples were run on the gel along with the control, which does not contain any template DNA. The expected bands were of either 400 bp or 100 bp. The markers used were in the range of 50 bp to 766 bp.

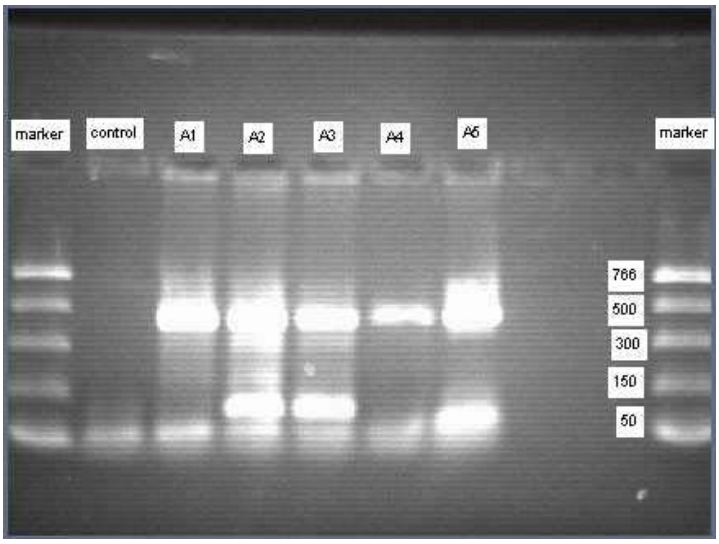

Figure 3.2: Alu TPA PCR gel electrophoresis. A1-A5 = Subjects. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

It can be seen that the two samples A2 and A3 are heterozygous for Alu of the tissue plasminogen activator gene, as they have one allele where Alu is present with a 400bp band and the other without Alu giving a 100bp band. However, the samples A1 and A4 seem to be homozygous as they only have a band of 400 bp. It is difficult to explain the bands obtained from sample A5 as there is a very intense band at 400bp but a more intense an extra primer dimer band at 50 bp than that found in A1-A4. Overall, it can be said that the extracted DNA is suitable for PCR and that the other reagents in the mix are functioning.

3.4 Optimisation of PCR

The summary of the actual PCRs undertaken for the respective gene regions is given below in table 6.

The primers were diluted to one fifth and one tenth of their initial concentration. Thus, when the PCR for a specific region found to be working, it did not work for all the samples used for the same reaction. That is why the reaction was repeated so many times with same DNA templates and with different samples as well. Not many PCRs for the intron regions of the gene were undertaken. The primers for intron PCR's were ordered towards the end of the term. Most of the session was consumed to get the exon PCRs working and given more importance.

S.NO.	Gene region amplified	No. of PCRs amplified	Outcome	
			Pass	Fail
1.	ALU-TPA	18	6	12
2.	5'FlankinG	8	2	6
3.	Exon 1- Intron A	8	1	7
4.	Exon 2 - Intron B – Exon 3	29	10	19
5.	Exon 3	32	13	19
6.	Intron C	10	3	7
7.	Exon 4	24	8	16
8.	3'FlankinG	9	7	2

Table7: Summary of the PCR reactions undertaken during the session

The sample A1 was used for all the gradient PCR's undertaken in the project. The Alu TPA PCR conditions were used with a annealing temperature gradient of

57±7°C. The numbers on the gel indicate the column number of the gradient PCR machine. The columns were at diiferent temperatures. The temperature in the gradient PCR machine from 6th column onwards was 57.8°C, 59.1°C, 60.5°C, 61.6°C, 62°C, 62.5°C and 63.2°C in the twelth column. Figures 3.3 to 3.9 show that the PCR for the three coding sequences of apo A1 gene have worked. The gradient PCR was run for the three coding regions to find out the correct annealing temperature.

3.5 Apo A1 5'flanking gradient PCR results

Expected amplicon size = 330 bp

The PCR product of the gradient PCR which amplifies *5*'flanking is shown in the figure 3.3. The standard PCR conditions were used with a annealing temperature gradient of 57±7°C. The strongest band could be seen in the 9th well. An extra primer band is present in every well at 50 bp. The annealing temperature for this region is 60.5°C.

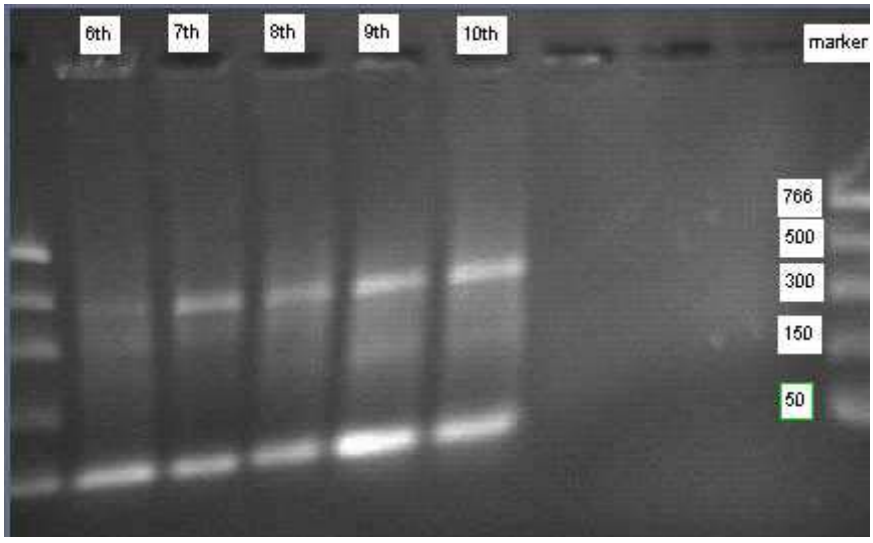

Figure 3.3: apo A1 5'flanking gradient PCR gel electrophoresis. 6-10 = wells of the gradient PCR machine. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp).

3.6 Exon 1/Intron A gradient PCR results

Expected amplicon size = 348 bp

The gradient PCR for the exon1/intronA was performed a couple of times but no bands were obtained. Therefore, the standard PCR conditions were used to run the PCRs for this region of the gene. The outome of the reaction on the gel is shown below in fig 3.4.

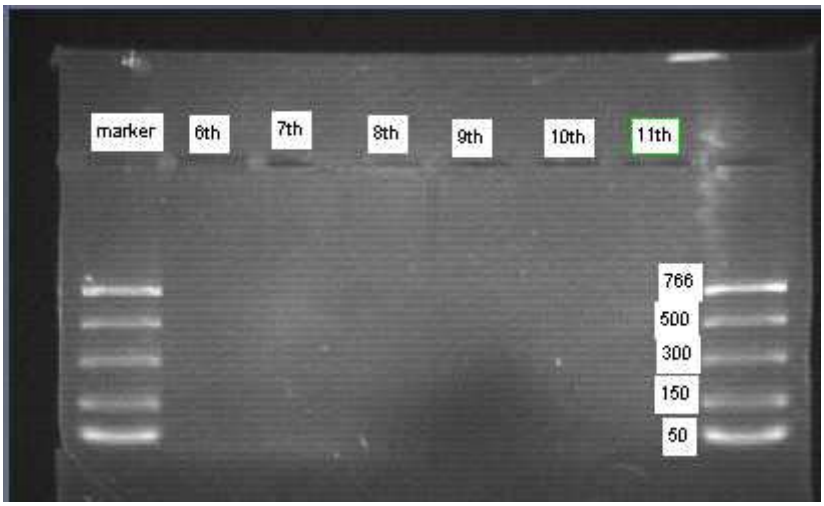

Figure 3.4: Exon 1/Intron A PCR gel electrophoresis. 6-11 = wells of the gradient PCR machine. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp).

3.7 Exon 2/Intron B/Exon 3 gradient PCR results

Expected amplicon size = 465 bp

The PCR product of the gradient PCR, which amplifies Exon 2 – Intron B and Exon 3, is shown in the figure 3.5. The control, which does not contain any DNA, was run along with all the samples. In 6th well the band is very pale but takes shape from the 7th well onwards. The strongest band of approximately 465 bp can be seen in the 10th well. So, the temperature in this well can be considered the annealing temperature (61.2°C). This temperature was used to run the future PCRs for this region.

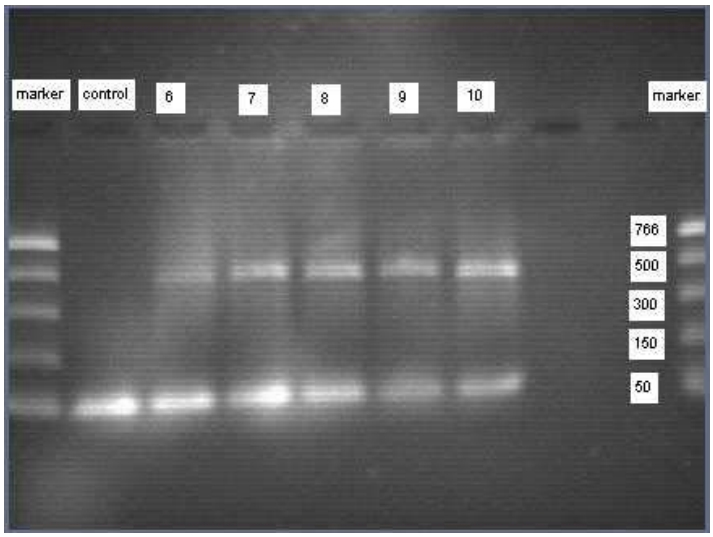

Figure 3.5: Exon2/IntronB/Exon3 gradient PCR gel electrophoresis. 6-10 = wells of the gradient PCR machine. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

3.8 Exon 3 gradient PCR results

Expected amplicon size = 209 bp.

The PCR product of the gradient PCR, which amplifies Exon 3, is shown in the figure 3.6.

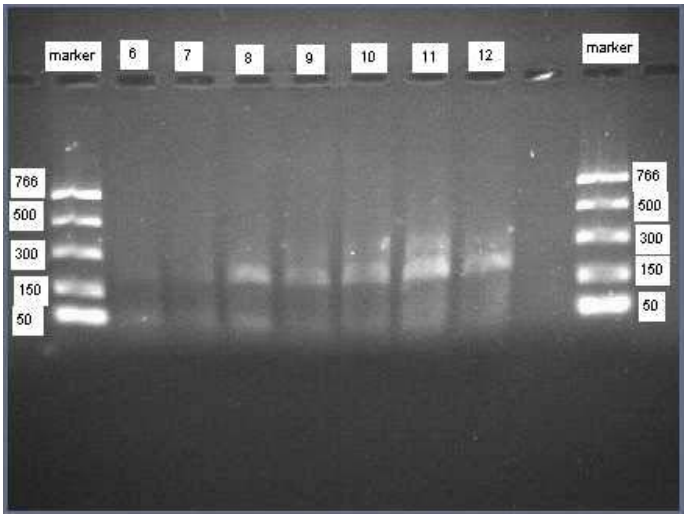

Figure 3.6: Exon 3 gradient PCR gel electrophoresis. 6-12 = wells of the PCR radient machine. The numbers on the left and right of the gel represent the size of the bands of the marker in base pairs (bp).

As mentioned earlier, the sample A1 was used to run the gradient PCR for this region. The Alu TPA PCR conditions were used with a annealing temperature gradient of 57±7°C. An extra primer-dimer band can be seen on 50 bp in every well. The strongest band of approximately 209 bp can be seen in the 11th well. This well was the correct annealing temperature (62.5°C) for the Exon 3 region.

3.9 Intron C gradient PCR results:

Expected amplicon size = 591 bp

The PCR product of the gradient PCR,which amplifies Intron C is shown in the figure 3.7. The standard PCR conditions were used with a annealing temperature gradient of 57±7°C. The strongest and a clear band of approximately desired size can be seen in 8th well. In this well was the correct annealing temperature (59.7°C). The bands in 9th and 10th well show a tail of bands. This could be due to the incorrect volume of the sample or process error. 59.7°C was the temperature used to run the future PCR's for this region.

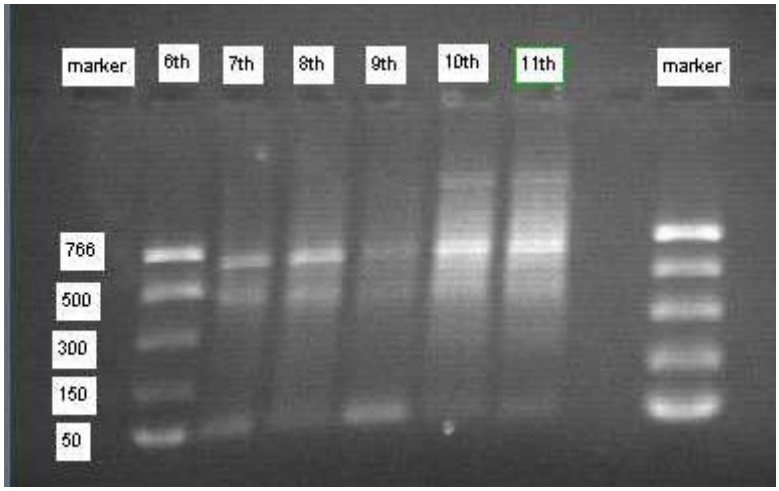

Figure 3.7 Intron C gradient PCR gel electrophoresis. 6-12 = wells of the PCR radient machine. The numbers on the left and right of the gel represent the size of the bands of the marker in base pairs (bp).

3.10 Exon 4 gradient PCR results

Expected amplicon size = 674 bp.

The PCR product of the gradient PCR, which amplifies Exon 4, is shown in figure 3.8. The sample A1 was used to run the gradient PCR for this region. The Alu TPA PCR conditions were used with a annealing temperature gradient of 57±7°C. The control, which does not contain any DNA, was run along with all the samples. The band in the 6th well is not clear while the strongest band of approximately 674 bp can be seen in the 7th well. In this well was the correct annealing temperature (58.1°C) for Exon 4 region.

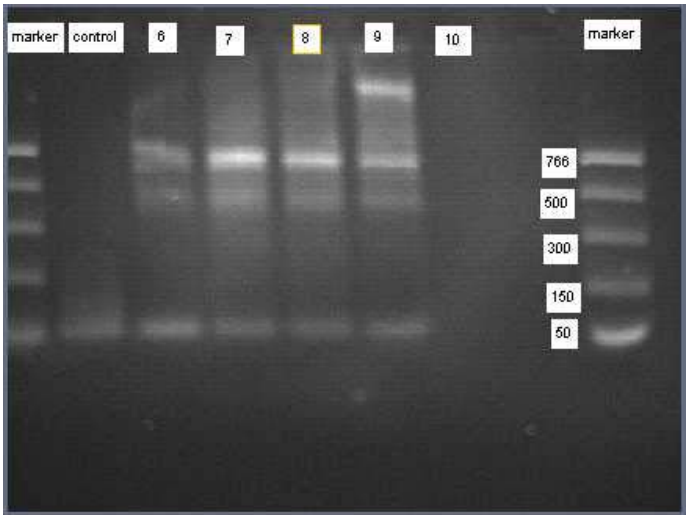

Figure 3.8: Exon 4 gradient PCR gel electrophoresis. 6-10 = wells of the gradient PCR machine. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

3.11 3'flanking gradient PCR results

Expected amplicon size = 364

The PCR product of the gradient PCR which amplifies Exon 1/ Intron A is shown in the figure 3.9. The control, which does not contain any DNA, was run along with all the samples. The standard PCR conditions were used with a annealing

temperature gradient of 57±7°C. The strongest band of approximately desired size could be seen in the 6th well. Therefore, the correct annealing temperature for this region was taken to be 57.8°C. All the bands have shown up with an extra primer dimer band at 50 bp.

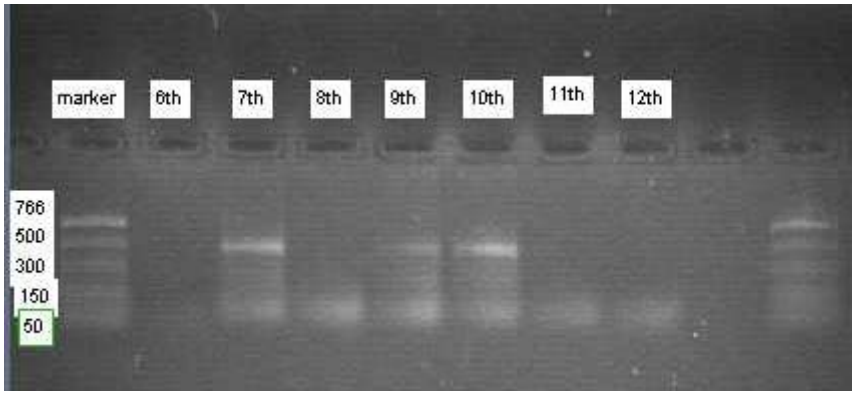

Figure 3.9: 3'flanking region gradient PCR gel electrophoresis. 6-10 = wells of the gradient PCR machine. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

Once the annealing temperature was determined, the polymerase chain reaction was performed with different DNA samples. Nine samples were used to run the following PCRs. Due to the actual number of PCRs undertaken, some samples were totally consumed and could not be used for every PCR. The results of these PCRs are presented below

3.12 Apo A1 5'flanking region PCR results

Expected amplicon size = 330 bp.

The PCR results for the apo A1 5'flanking region are shown in Fig 3.10. Eight of nine samples were run on the gel. A very clear band can be seen of approximately 330 bp in the 5th well (sample A5) and a less intense band for samples A1, A3, A4, A7 and A8. Samples A2 and A6 have not produced any bands at all.

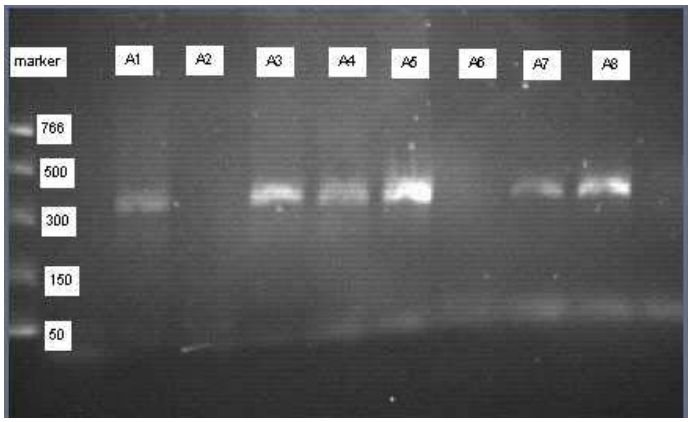

Figure 3.10: apo A1 5'flanking region PCR gel electrophoresis. A1-A8 = subjects the numbers on the left of the gel represent the size of the bands of the marker in base pairs (bp).

3.13 Exon1 / Intron A PCR results

Expected amplicon size = 348 bp.

The PCR results for exon1 / intron A region are shown in figure 3.11. The control was run along with all of the samples. The standard PCR conditions were used for this PCR. So, the annealing temperature for this PCR was 57ºC. Only three samples A1, A4 and A9 worked for this PCR and showed bands of approximately 348 bp.

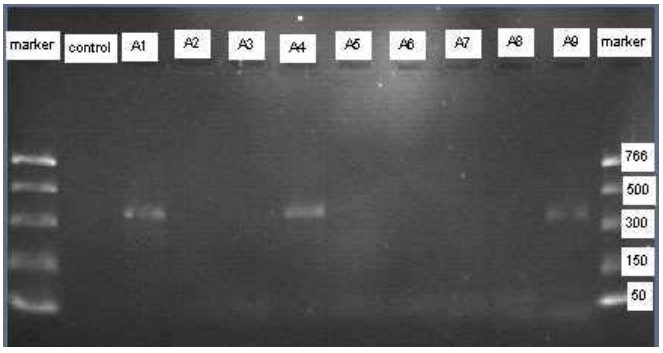

Figure 3.11: Exon1/Intron a PCR gel electrophoresis. A1-A9 = subjects. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

3.14 Exon2 / Exon3/ Intron B PCR results

Expected amplicon size = 465 bp.

The PCR results for exon2 / exon3/ intron B are shown in the figure 3.12. The annealing temperature for this PCR was 61.2 ºC.

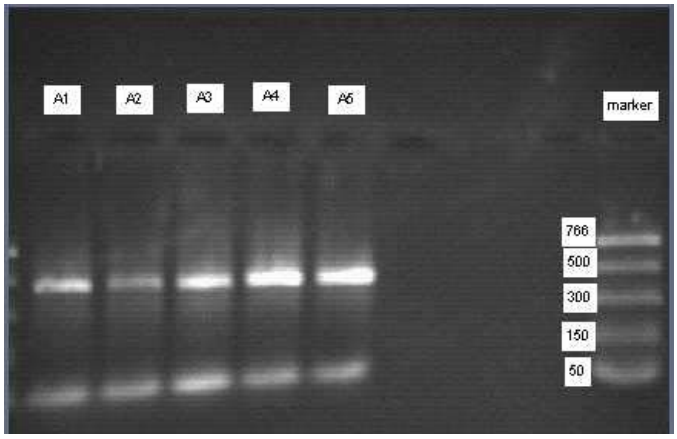

Figure 3.12: Exon1/Exon2/Intron B PCR gel electrophoresis. A1-A5 = subjects. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp).

Five out of nine samples were run on the gel. The marker bands on the left hand side have not been clearly captured in the gel picture. Samples A3, A4 and A5 have given a very strong band of approximately 465 bp.,whereas, samples A1 and A2 are comparatively faint. An extra primer-dimer can be seen at 50 bp for all of the samples.

3.15 Exon 3 PCR results

Expected amplicon size = 209 bp.

The PCR results for exon3 region are shown in Fig 3.13. The annealing temperature for this PCR was 62.5ºC. Six out of nine samples were run on the gel.

The control, which does not contain any DNA, was run on the both ends of the gel along with all the other samples. It can be seen that the bands of approximately 209 bp are apparent in the samples A1, A2, A3 and A5. There is also an extra product present at 300 bp. Samples A4 and A6 have not worked at all.

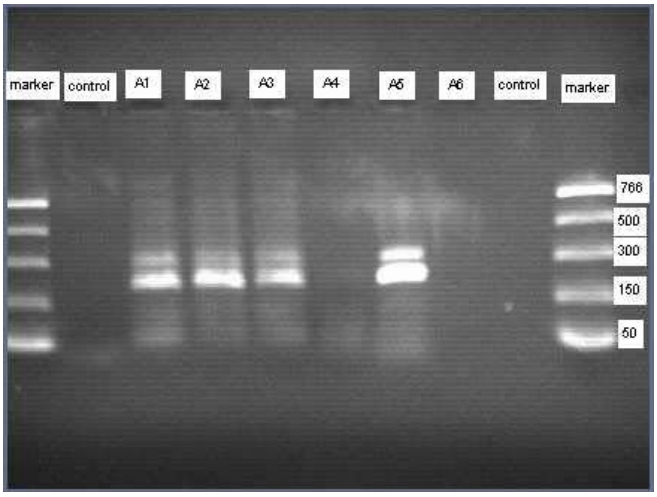

Figure 3.13: Exon3 region PCR gel electrophoresis. A1-A6= subjects. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

3.16 Intron C PCR results

Expected amplicon size = 591 bp.

The PCR results for intron C region are shown in figure 3.14. The annealing temperature for this PCR was 59.7ºC. The control, which does not contain any DNA, was run along with all the other samples. The strongest band of approximately 591 bp can be seen with a trail for sample A9. Samples A1, A3 and A8 have given bands with a trail as well. Samples A2, A4, A5, A6 and A8 did not work. A faint primer-dimer can be seen for all of the samples.

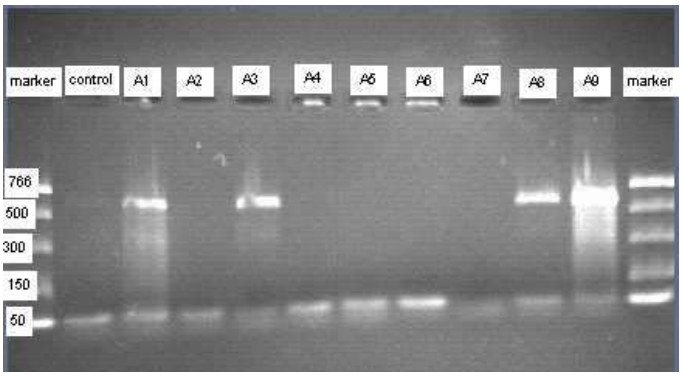

Figure 3.14: Intron C region PCR gel electrophoresis. A1-A9 = subjects. Control = no template DNA present. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp).

3.17 Exon 4 PCR results

Expected amplicon size = 674 bp.

The PCR results for exon 4 region are represented in the figure 3.15. The annealing temperature for this PCR was 58.1ºC. Four out of nine samples were run on the gel. The control, which does not contain any DNA, was run along with all the other samples. Samples A1, A2 and A3 have given very clear bands of approximately 674 bp along with a fainter band at about 500 bp. But the bands show a trail all along their way in the gel. Sample A4 shows a very faint band. There is an extra primer-dimer band present at 50 bp.

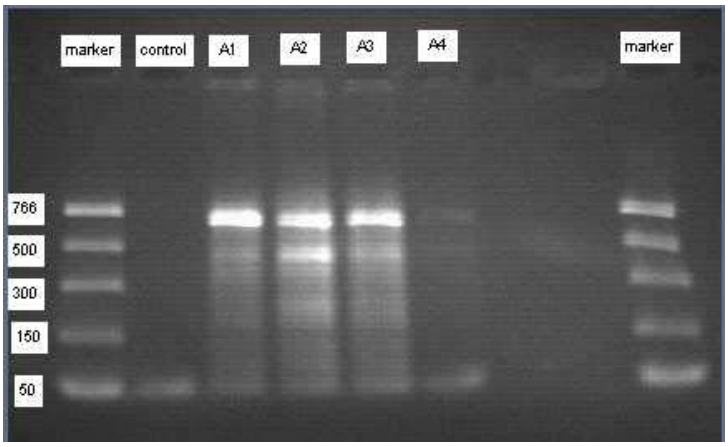

Figure 3.15: Exon 4 region PCR gel electrophoresis. A1-A4 = subjects. The numbers on the left of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

3.18 Apo A1- 3'flanking PCR results

Expected amplicon size = 364 bp.

The PCR results for the apo A1 3'flanking region are shown in figure 3.16. the annealing temperature for this PCR was 57.8ºC The control, which does not contain any DNA, was run along with all the other samples. Only samples A1, A3 and A9 have given the desired band of approximately 364 bp. Addittionaly there is a faint band at about 500 bp in samples A1, A3 and A9. The primer-dimer bands at 50 bp can also be seen and are very faint.

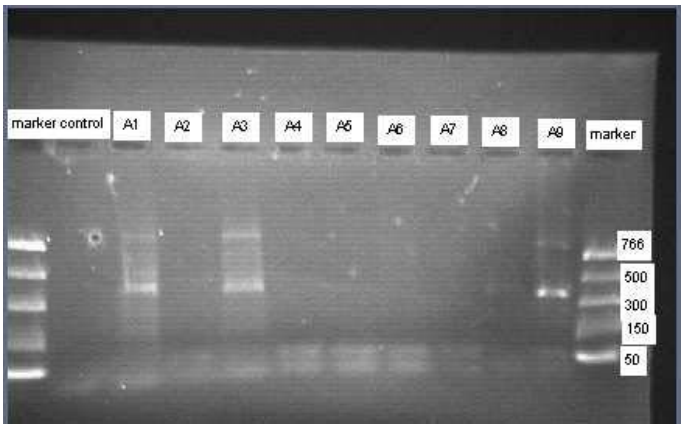

Figure 3.16: apo A1 3'flanking region PCR gel electrophoresis. A1-A9 = subjects. The numbers on the right of the gel represent the size of the bands of the marker in base pairs (bp). Control = no template DNA present.

A calibration curve relating distance moved to the size of the marker agraph can be obtained (figure 3.17) from the marker lanes. By measuring the distance moved by the unknown bands on the gel, their actual size can be estimated.

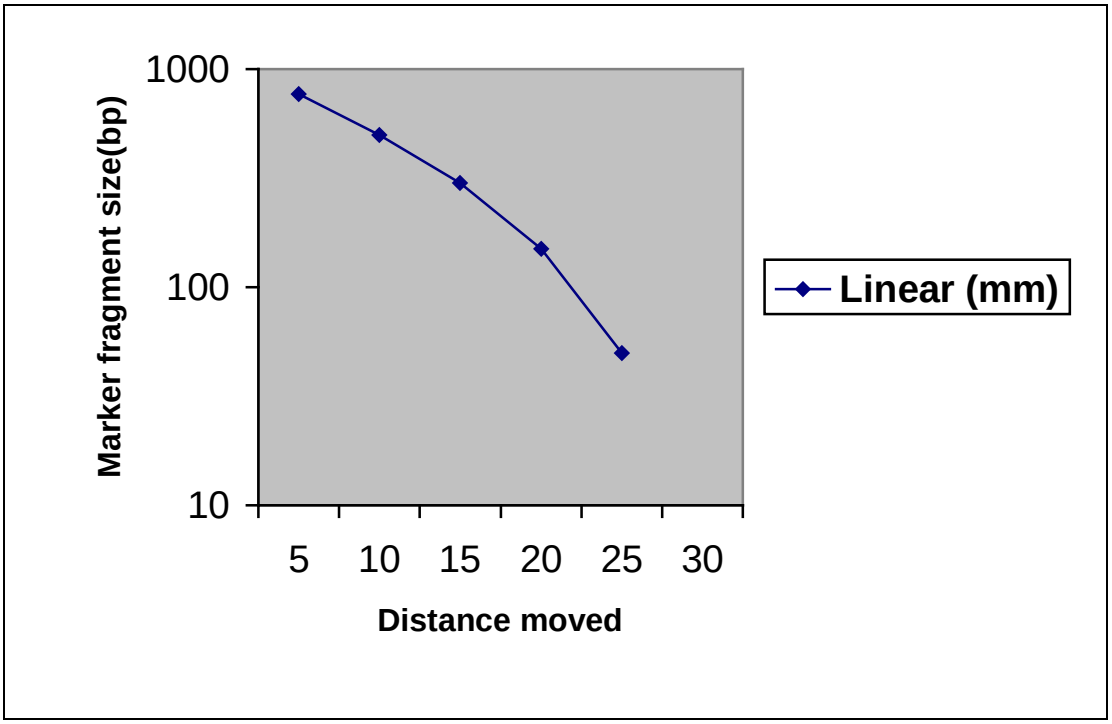

Figure 3.17: Semi-log graph of fragment size against the distance moved by the marker.

Chapter 4
Sequencing

4.1 Sequencing

60 samples were sent for sequencing. The samples were sequenced with both forward and reverse primers. Not all the samples were sequenced successfully with the forward and reverse primer for the same sample. Reverse sequences were converted to reverse complements to be compatible with the forward sequences. The sequences were edited in the Bio-Edit program and exported into the Fasta format. The edited sequences were then aligned with the original database gene sequence in CLUSTAL W. Lack of alignment of sequences will usually involve miscalling of bases, each sequence should therefore be run five or six times on each sample. That was not the case here. Polymorphisms should be consistent variants in a sample. There was insufficient time and resources to cover all statistical probabilities. The summary of the actual samples sent for sequencing for the respective region of the gene are listed below in the table 7.

S.no	Gene region	Number of samples sequenced	outcome	
			Pass	Fail
1.	5'Flanking	6	5	1
2.	Exon 1- Intron A	2	--	2
3.	Exon 2 - Intron B – Exon 3	16	5	11
4.	Exon 3	16	3	13
5.	Intron C	2	--	2
6.	Exon 4	12	3	13

7.	3'Flanking	6	--	6

Table 8: summary of the samples sequenced during the term

Successful sequencing results are represented below according to the different regions of apo A1 gene.

4.2 Apo A1 5'flanking region

The sequence of the apo A1 5'flanking region is given below. The bold letters refers to the nucleotide where the polymorphism or a miss calling of base may be present.

```
  1  tctccctgga atgctggtgg tgggggaggc agtctccttg gtggaggagt cccagcgtcc
 61  ctccctccc ctcctctgcc aacacaatgg acaatggcaa ctgcccacac actcccatg
121  aggggaagg gatgagtgca gggaaccccg accccacccg ggagacctgc aagcctgcag
181  acactcccct cccgccccca ctgaaccctt gacccctgcc ctgcagcccc cgcagcttgc
241  tgtttgccca ctctatttgc ccagccccag ggacagagct gatccttgaa ctcttaagtt
301  ccacattgcc aggaccagtg agcagcaaca
```

The Clustal multiple alignment is shown in figure 4.1 for the samples sequenced with the forward primer of apo A1 5'flanking region. There is an insertion of base G at position 131 in all the samples, which is not present in the original gene sequence. The reference sequence of of apo A1 5'flanking region in the clustal alignment can be matched with the original gene sequence of the gene. Because of the miscalling bases or sequencing error at positions 131, 149, 150, 162 and 183, the position of the bases has moved in the alignment. The sample A8 shows an apparent insertion of bases A, G, T and T at positions 146, 149, 162 and 184, respectively. In sample A7 alignment, the nucleotide C is replaced by T at position 169 and an insertion of nucleotide T at position 184.

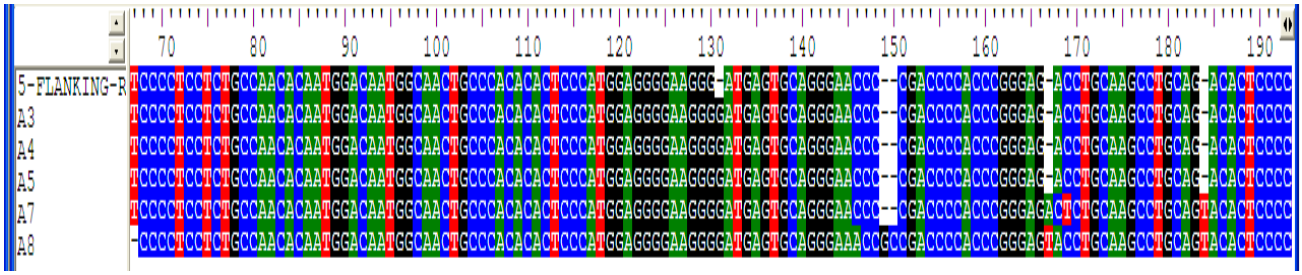

Figure 4.1 Clustal multiple alignment of apo A1 5'flanking region (forward primer sequencing).

4.3 Exon 2/ Intron B/ Exon 3 Region

The gene sequence of Exon 2/ Intron B/ Exon 3 region is given below.

```
581 catgctgaag
591 gcaccccact cagccaggcc cttcttctcc tccaggtccc ccacggccct tcaggatgaa
651  agctgcggtg  ctgaccttgg  ccgtgctctt  cctgacgggt  aggtgtcccc  taacctaggg
711 agccaaccat cgggggggctt tctccctaaa tccccgtggc ccaccctcct gggcagaggc
771  agcaggtttc  tcactggccc  cctctccccc  acctccaagc  ttggcctttc  ggctcagatc
831  tcagcccaca  gctggcctga  tctgggtctc  ccctcccacc  ctcagggagc  caggctcggc
891  atttctggca  gcaagatgaa  ccccccccaga  gcccctggga  tcgagtgaag  gacctggcca
951 ctgtgtacgt ggatgtgctc aaagacagcg gcagagacta tgtgtcccag tttgaaggct
1011 ccgccttggg aaaacagcta aagtaaggac ccagcct
```

The Clustal multiple alignment is shown in figure 4.3 for the samples sequenced with the forward primer of exon 2/ intron B / exon 3. The samples A3, A4, and A5 were sequenced successfully and show no variation from the original gene sequence until base number 929. Where as, the sequencing for samples A1 and A2 were failed partially.

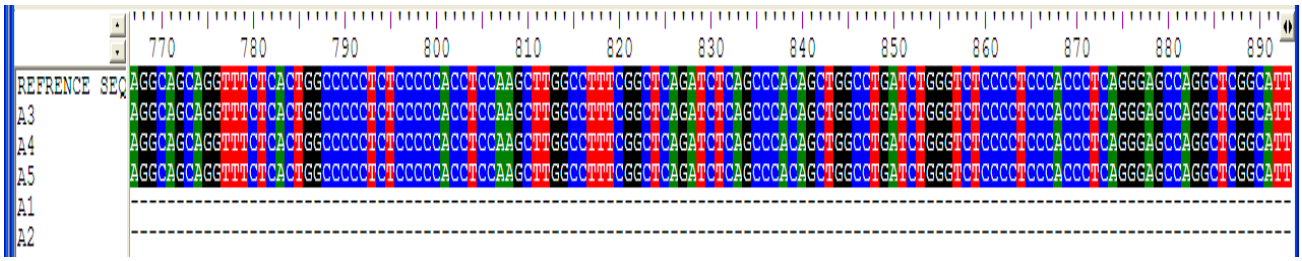

Figure 4.3 Clustal multiple alignment of exon2/intronB/exon3 region (forward primer sequencing).

The variations in the sequences in Clustal multiple alignment of exon2/intronB/exon3 is shown in the figure 4.4. There is a miscalling of a base at position 930 for all the samples including the reference sequence. There is an apparent insertion of nucleotide A at position 985 in sample A5. In sample A3 alignment, the nucleotide G is replaced by T at position 988 and it shows an insertion of nucleotides T and C at positions 1028 and 1042, where as there is an insertion base G at the same positions in sample A4 and A5. Due to these variations in the sequences the base positions have moved and different from the original gene sequence.

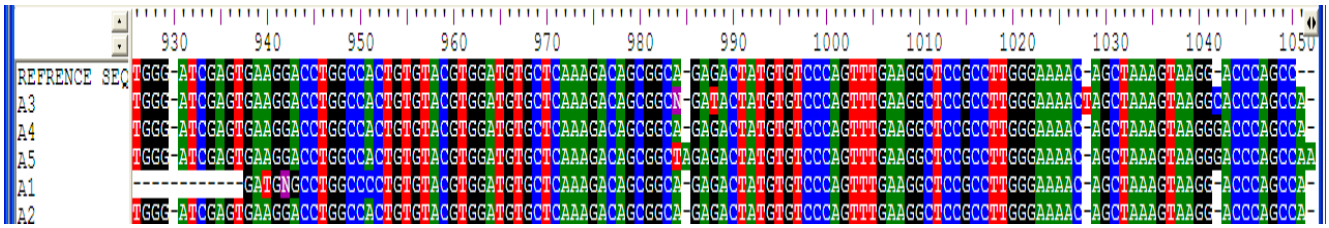

Figure 4.4: Clustal multiple alignment of exon2/intronB/exon3 region (forward primer sequencing).

The reverse complement of the gene sequence of Exon 2/ Intron B/ Exon 3 region is given below.

```
ggctgggtcc ttactttagc tgttttccca aggcggagcc ttcaaactgg gacacatagt

ctctgccgct gtctttgagc acatccacgt acacagtggc caggtccttc actcgatccc
```

aggggctctg ggggggttca tcttgctgcc agaaatgccg agcctggctc cctgagggtg

ggaggggaga cccagatcag gccagctgtg ggctgagatc tgagccgaaa ggccaagctt

ggaggtgggg gagaggggggc cagtgagaaa cctgctgcct ctgcccagga gggtgggcca

cggggattta gggagaa**a**gc cccccgatgg ttggctccct aggttagggg acacctaccc

gtcaggaaga gcacggccaa ggtcagcacc gcagctttcat cctgaagggc cgtgggggac

ctggaggaga agaagggcct ggctgagtgg ggtgccttcag cat

The Clustal multiple alignment is shown in figure 4.5 for the samples sequenced with the reverse primer of exon 2/ intron B / exon 3. A lot of variations could be seen in the sample A5. There is an apparent insertion of nucleotides A, G, A, A, T, C, A, C, A, G at positions 717, 718, 728, 733, 749, 755, 756, 763, 781 and 782 respectively.

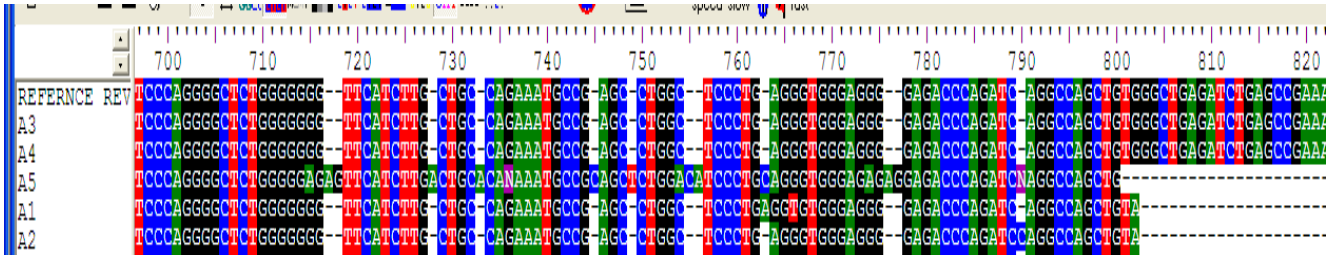

Figure 4.5 Clustal multiple alignment of exon2/intronB/exon3 region (reverse primer sequencing).

4.4 Exon 3 region

The gene sequence of Exon 3 region is given below.

871 ctcagggagc caggctcggc

891 atttctggca gcaagatgaa ccccccccaga gcccctggga tcgagtgaag gacctggcca

951 ctgtgtacgt ggatgtgctc aaagacagcg gcagagacta tgtgtcccag tttgaaggct

1011 ccgccttggg aaaacagcta aagtaaggac

The forward primer sequencing of exon 3 failed. The reverse complement of the gene sequence of Exon 3 region is given below.

cccaggctgg gtccttactt tagctgtttt cccaaggcgg agccttcaaa ctgggacaca

tagtctctgc cgctgtcttt gagcacatcc acgtacacag tggccaggtc cttcactcga

tcccaggggc tctgggggggg ttcatcttgc tgccagaaat gccgagcctg gctccctgag

ggtgggaggg gagacccaga

The clustal multiple alignment is shown in figure 4.6 for the samples sequenced with the reverse primer of exon 3. Samples A1 and A2 show insertions of nucleotides at various positions throught out the sequence as compared to the reference reverse complement sequence of exon 3.

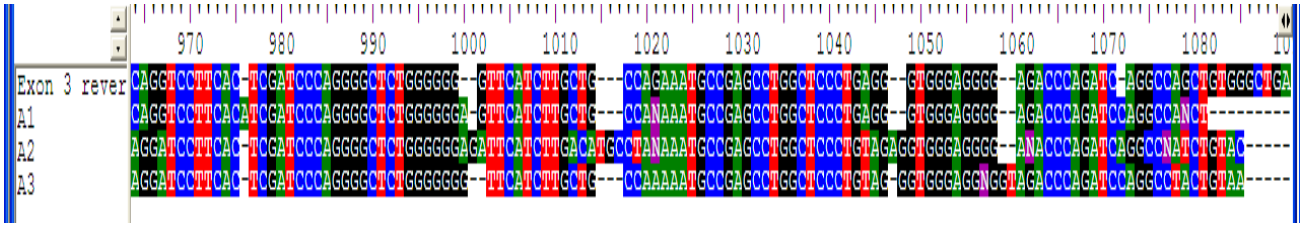

Figure 4.6 Clustal multiple alignment of exon3 region (reverse primer sequencing).

4.5 Exon 4 region

The gene sequence of Exon 4 region is given below.

1591 cagccctcaa cccttctgtc

1611 tcaccctcca gcctaaagct ccttgacaac tgggacagcg tgacctccac cttcagcaag

1671 ctgcgcgaac agctcggccc tgtgacccag gagttctggg ataacctgga aaaggagaca

1731 gagggcctga ggcaggagat gagcaaggat ctggaggagg tgaaggccaa ggtgcagccc

1791 tacctggacg acttccagaa gaagtggcag gaggagatgg agctctaccg ccagaaggtg

1851 gagccgctgc gcgcagagct ccaagagggc gcgcgccaga agctgcacga gctgcaagag

1911 aagctgagcc cactgggcga ggagatgcgc gaccgcgcgc gcgcccatgt ggacgcgctg

1971 cgcacgcatc tggcccccta cagcgacgag ctgcgccagc gcttggccgc gcgccttgag

2031 gctctcaagg agaacggcgg cgccagactg gccgagtacc acgccaaggc caccgagcat

2091 ctgagcacgc tcagcgagaa ggccaagccc gcgctcgagg acctccgcca aggcctgctg

2151 cccgtgctgg agagcttcaa ggtcagcttc ctgagcgctc tcgaggagta cactaagaag

```
2211 ctcaacaccc agtgaggcgc ccgccgccgc ccccttccc ggtgctcaga ataaacgttt
```

The Clustal multiple alignment is shown in figure 4.7 for the samples sequenced with the forward primer of exon 4. The samples A1 and A2 show an apparent insertion of nucleotide T at positions 1776, 1778, 1789, 1790, 1821, 1839, 1843, 1857, 1861 and 1878. In sample A1 and A2, the nucleotide A and T are missing at positions 1793 and 1832 respectively.

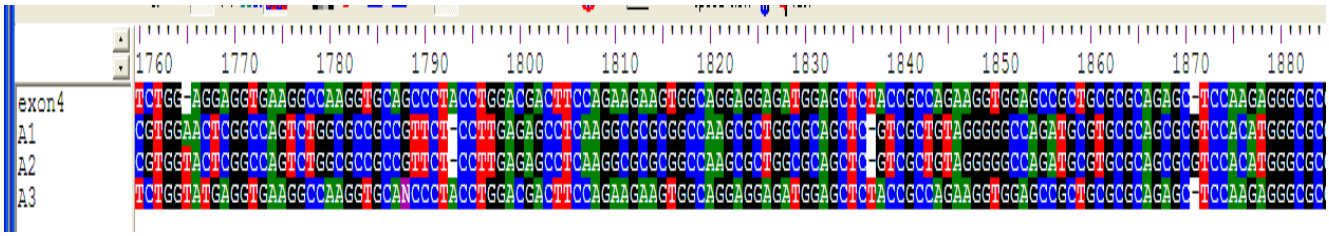

Figure 4.7 Clustal multiple alignment of exon 4 region (forward primer sequencing).

Chapter 5
Discussion

5.1 Discussion

The aim of the project was to devolp PCR based methods for the detection of polymorphisms in the human Apo A1 gene of normal individuals. The specific objectives included:

1. Using Bioinformatics tools, to define appropriate regions of the gene for PCR amplification.
2. To optimise exon, intron and control region PCRs to provide amplicons for sequencing.
3. To sequence amplicons in groups to identify potential polymorphisms.
4. To use data from 3. In the development of AFLP (amplified fragment length polymorphism) or ASO (allele specific oligonucleotide) methods for rapid detection of polymorphism.

The project arises form an existing PhD project in which damaged (nitrated) proteins were identified from the plasma of claudicants (*Heptinstall, J. et al, 2006*). The nitrated proteins found in these patients (who suffer from severe lower limb atherosclerosis making walking difficult) and also in the controls did not yield an obvious biomarker for the disease. However, one of the proteins was apolipoprotein A-1 (Apo A1) which has a well known role in atherosclerosis but which can change if the protein changes.

Apo A1 is the major protein of HDL and therby promotes cholesterol efflux from the tissue to the liver for extraction; thus its role is anti atherogenic. However there are variants of the APO A1 that are either more anti or even pro-atherogenic in relation

the the wild type allele. These variants in the coding regions (exons) of the gene are quite rare. The best known anti-atherogenic mutation, known as Apo A1 (Milano) was found over 20 years age in a few families in Limone on Lake Grada – today there are 36 individuals with this allele – where it confers protection against atherosclerosis and consequent cardiac disease (*Parolini, C. et al, 2005*). In the main though, variant alleles are pro-atherogenic but not common. Thus screening for amino acids substitutions in the Apo A1 of 32,000 new borns in Germany revealed 16 variants (1 in 2000) in some of which the Apo A1 (and HDL) concentration was decreased (*Eckardstein, A. et al, 1989*). Also in Germany, 1 in 750 of a randomly screened population of 2,282 showed a pro-atherogenic mutation, Apo A1Marburg (*Utermann, G. et al, 1982*), whilst only 3 cases of mutation leading to Apo A1 deficiency have been described from western Jpan (*Sasaki, J. et al, 2000*).

Mutations in the non coding regions of the gene are more common, but not normally associated with Apo A1 deficiency and disease. Thus an Sst-1 RFLP in the 3' flanking region was found in 1 in 20 people in the UK but with no effect on Apo A1 levels (*Rees, A. et al, 1985*).

Since there was no biomarker nitrated protein found in claudicants that was not also present in controls, it is possible that the claudicants may have a pro-atherogenic variant of Apo A1. The proposed project therefore aimed to establish the methods, in control subjects, that can be applied to claudicant samples at a later date. It was not anticipated that any polymorphisms would be found in the exons; there is a<5% probablility of finding a polymorphism in the less significant and flanking regions.

Many studies have been undertaken to find mutations in the apo A1 gene. Most of them showed variation in the primary structure of the protein resulting from differences in the sequence of the gene. All mutations were found in people who already had some clinical symptoms. This is why the people without mutations only have lower probability of developing the disease.

The gene products from the *apolipoprotein (APO) A1/C3/A4/A5* gene cluster (11q23–24) are involved in lipid metabolism. Apolipoprotein (apo) A1 comprises approximately 70% of the high-density lipoprotein (HDL) particle. It acts as a cofactor of lecithin-cholesterol acyltransferase in facilitating the transport of cholesterol from the peripheral tissues to the liver in a process known as reverse cholesterol transport. The statistical associations between variation in plasma levels of HDL-C, TC and TG and single-site and haplotype variations in the apo A1 gene have clearly established the influence of this gene in determining phenotypic variability. The present study was undertaken to investigate the reproducibility of the apo-A1 gene sequence in normal healthy Caucasian control individuals from Coventry University, UK for a better assessment of role of apo A1 mutations in conferring risk of artherosclerosis in claudicants. Some apparent gene variations were found. It is however difficult to make any inference how these variations could be associated with artherosclerosis in claudicants.

The main reason for this was the small sample size which was further reduced due to failed sequencing reaction in some samples. We did not identify any novel mutations in the sample studied and some of these mutations primarily the one in intronic region are known to be commonly polymorphic. True estimates cannot be

made of the allele and genotype frequencies of the polymorphisms that we observed in our small sample size. However our preliminary study provides the basis for further investigation of the entire spectrum of mutations in the apo A1 gene. It will be interesting to address the following questions in further study.

What is the gamut of rare mutations in apo A1 that could possibly explain some high penetrance effects of this gene in predisposition to artherosclerosis in claudicants? Are there any population specific disease risk alleles that enhance risk?

The Alu TPA PCR was performed during the first phase of the project. And, the master mix from this PCR was also used for the detection of the correct annealing temperature of different coding and non-coding regions of apo A1 gene. The PCR conditions used for the Alu TPA PCR are the standard PCR conditions to begin with. That is why the same conditions were used to detect the annealing temperature of the apo A1 gene regions. As mentioned in the results section, additional bands were seen on every gel of the gradient PCR. In gradient PCR the reactions which were not optimised gave the non specific DNA products or did not produce any any amplified products at all. Once the annealing temperature was detected, the PCRs for all the samples were performed. But the desired PCR products were not obtained from all of the DNA samples. At times, non specific or no PCR products were obtained. The reasons may be the different concentrations or the purity level of the samples.

DNA purification could be another reason of PCR failure. As it has been mentioned in earlier sections, the A 260/280nm ratio was not even close to the value of 2 for all the subjects. Sample A1 has A260/280nm ratio of 1.89. This sample has given a product

band in almost every reaction. Where as other samples sometimes worked in one reaction and sometimes not. The A260/230nm ratio was low for each sample. Apart from any human errors the value of this purification ratio needs to be considered everytime a PCR is done.

After the gradient PCRs, the clear bands from the eight subjects were expected. But, it did not happen for each and every sample. The exon 3 PCR results (figure 3.13) show very bright bands of approximately 300bp for samples A1, A2, A3 and A5. But, samples A4 and A6 failed to produce any bands at all. The concentration of the DNA templates varied from 24.3ng/µl (A4) to 104ng/µl (A1).

The concentration of sample A5 is 37ng/µl. There is not much difference between the concentration of sample A4 and A5 as that of sample A1 (104 ng/µl) and A5. The DNA purification level is more for sample A4 (A260/280=1.47) than that of sample A5 (1.31). Either a measurement error had occurred for sample A4 or a human error during the preparation of PCR. The PCR's of the non-coding regions of apo A1 gene did not give any sound results.

The sequencing reactions were not successful. All the samples could not be sequenced for their reverse and forward primers. Some falied for both of the primers and some worked for forward and some for reverse primers. This was the major variable in the process to draw any inferences of any polymorphisms present in the study of this small sample size. The sequences show frequent miscalling of the bases. The insertion of various nucleotides could be seen at various places in a sequence in all the samples. At most places it was the sequencing error. Intron regions of the gene were not sequenced successfully. The miscalling of bases in the sequencing reaction on an

average is 5 per 100 bases. There is not enough data to compare the results of introns and exons.

Cross contamination could be another reason for PCR to failure. In the beginning of the project none of the PCRs seemed to be working. It was later on revealed that there could be a chance of contamination of the DNA template. Since then, a negative control sample, which does not contain and DNA template, was run along with all the other samples. The primers, PCR buffer and other solutions used for the reaction could be cross contaminated. Eventually, after making fresh PCR buffer solution and running control samples, a lot of improvement was seen in the PCR results. The other reason could be the level of DNA purification ratio. The DNA isolation protocol was followed with all precautions. An absorbance 260/280 nm ratio of 1.8 could not be achieved for any sample. This needs to be amended for the future PCR work. The concentration of the primers was changed to achieve good PCR results. The concentration was lowered to one fifth and one tenth of their original. Irrespective of the sample concentration, 5µl of the DNA template solution was used for every reaction. This was the major variable in the procedure. This should be avoided in future PCR work by calculating the amount of template DNA.

Multiple repetition of PCR has to be run in order to find any differences in the sequences. This should be done with the patients who already have developed clinical symptoms of atherosclerosis, because otherwise a mutation is unlikely. It can be said that the PCR program did not happen to work with every DNA sample. It is expected that the higher the DNA amount is the stronger is the PCR product on the gel. Because, more ethium bromide can bind to more available DNA and consequently the resulting

fluorescence is more intensive. But some samples with higher concentration failed to produce such kind of bands, which might be due to any human errors or the failure of program. Sample A1 (104ng/ml) and A2 (61.6ng/ml) were the high yielding samples. They showed the product band in almost every reaction.

Taq-polymerase is a thermostable polymerase used in the PCR to generate the amplicon. One unit of *Taq* was used in every reaction. One of its drawbacks is the low replication fidelity as it lacks a 3' to 5' exonuclease proofreading mechanism. The error rate of taq polymerase is 1 in 9000 nucleotides. But it seems that either the error rate of the taq is quite high in the process or it has to do with the purification of DNA. Taq Man real time PCR method could be used to overcome this. It would help to measure the accumulation of the product at exponential stages of the reaction. Apparently, the focus of the project was not on the expression of apo A1 gene.

Other techniques like quantitative PCR could be used in the future to quantify either the starting amounts of DNA template or at the end of the reaction (end point assay). When the amount of DNA template is measured during the PCR steps is called as Real Time PCR.

When there is a difference in the DNA sequence in a population, or the occurance of two or more genetically determined phenotypes together in such a manner that the rarest of them cannot be maintained merely by recurrent mutation, it gives rise to a polymorphism. It may be induced by an external agent such as virus or radiation .And if the DNA sequence is found to be associated with any disease is called as genetic mutation. Any locus with more than one allele is polymorphic.

Mutation is change in DNA sequence from normal. Polymorphism if they exist can be SNP, insertion, deletion anywhere in the gene. The different types are explained as follows:

Single nucleotide polymorphism (SNP)

It is the most common type of polymorphism. In this type of variation the single base differs between individuals. For example, A is replaced by G. it generally occurs in the gene and the surrounding area of genome which control their expression. It may not have a very large effect but still it influences the activity of the encoded protein (Strachan, T and Read, A. 1996).

Restriction fragment length polymorphism (RFLP)

It is a technique in which organisms may be differentiated by analysis of patterns derived from the cleavage of their DNA. If two organisms differ in t he distance between the cleavage of a particular restriction endonuclease , the length of the produced fragments will differ when DNA is digested within a restriction enzyme. The pattern can be used to segregate species from one another.

Restriction endonucleases are the enzymes which break DNA molecules at a particular nucleotide depending on the type of enzyme used. it is basically derived from bacteria. The number of produced fragments depends on the length of the sequence. More number of fragments is generated with the shorter sequence. And so often the different sizes of the fragments are produced if the molecules have different nucleotide. This can further be separated by gel electrophoresis.

RFLP can be used to detect the diseased gene in the body by the domain of its presence or absence. According the concept of Crossing Over RFLP may get linked to a mutant gene in some people that's why all the healthy people are supposed to undergo testing as well who are living the subjects. They may be either homozygous or heterozygous (Strachan, T and Read, A. 1996).

Generally the mutations are not associated with a disease but rather they are present in the noncoding DNA sequences. As a large number of recognition sequences are known for type 2 restriction endonucleases , many point mutations polymorphism will be characterised by alleles which possess or lack a recognition site for a specific restriction endonuclease and therefore display restriction site polymorphism(RSP). The two individuals under the same species range might have the same genome but they differ in their nucleotides and the restriction sites. So, therefore, two detectable alleles with a lacking and possessing specific restriction site are present in the restriction site polymorphism.

Besides following a particular sequence of DNA, RFLP can be used to determine source of DNA in criminal or paternity cases and measurement of recombination rates which further helps to build the genetic maps of chromosomes.

VARIABLE NUBER OF TANDEM REPEATS (VNTR)

The sequences of DNA in the chromosomes are repeated a number of times, precisely known as tandem repeats. DNA probing is used to detect the VNTR polymorphism within the allele samples having different VNTR. For this, DNA is acted upon by restriction endonucleases that will find a restriction site with a VNTR locus. The fragments produced are segregated by gel electrophoresis and then hybridised to from

the respective locus. The different sizes of the restriction sites present the number of repeated unit. Usually the repeat lengths are considered in the 5-64bp region which differentiate it from the microsatellite DNA or the simple tandem repeat polymorphism. If the VNTR locus is from repeated DNA fragments then complex polymorphic pattern is produced rather than a unique one (Strachan, T and Read, A. 1996).

5.2 Conclusion

LDL cholesterol has been given the prime consideration for a long time. But now for the last two decades, the researchers have started giving emphasis on the low levels of HDL cholesterol, which is equally important because of the presence APO A1and relation to certain cardiovascular diseases. Low HDL is atherosclerotic marker. ABCA1 has been found to be involved in the first step of cholesterol efflux, so it has started gaining importance in the new researches to give the additional protection towards atherosclerosis. The PCR procedure used in the project was successful for the amplification of all the exons and introns of apo A1 gene. Nevertheless, further work is needed to improve the the amplification procedure. The sample size should be big enough to make any conclusions for any polymorphisms present. The sequencing results do have many errors which can not be avoided and have to manually edited. There are certain details which need to be worked out such as:

- PCR thermal profile (primer annealing, extension, denaturation, ramp times and number of cycles.
- Magnesium ion concentration
- Primer design and concentration
- Template quality and concentration

- PCR buffer
- dNTP concentration
- Addition of PCR enhancers
- Avoidance of PCR inhibitors
- Secondary PCR with nested primers
- Hot start PCR

The sequencing results showed amplicons of shorter length than expected. The sequencing was done directly from the end of each amplicon. Better results can be achieved with sequencing starting in the strand and not at the end. These kinds of strands can be gained with nested PCR, where the product of the first PCR is used in the second PCR. Thereby, the unwanted or a wrong product of the first PCR would be eliminated.

Chapter 6
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